University of Bath

PHD

Activation of signal transduction pathways by monocyte chemotactic proteins-1, -2, -3 and -4 in the THP-1 monocytic cell line: regulation of CCR2 signalling cascades by phosphatidylinositol-3-kinases

Logan, Marisa Kay

Award date:
2002

Awarding institution:
University of Bath

Link to publication

Alternative formats
If you require this document in an alternative format, please contact:
openaccess@bath.ac.uk

Copyright of this thesis rests with the author. Access is subject to the above licence, if given. If no licence is specified above, original content in this thesis is licensed under the terms of the Creative Commons Attribution-NonCommercial 4.0 International (CC BY-NC-ND 4.0) Licence (https://creativecommons.org/licenses/by-nc-nd/4.0/). Any third-party copyright material present remains the property of its respective owner(s) and is licensed under its existing terms.

Take down policy
If you consider content within Bath's Research Portal to be in breach of UK law, please contact: openaccess@bath.ac.uk with the details. Your claim will be investigated and, where appropriate, the item will be removed from public view as soon as possible.

Download date: 05. May. 2023

Activation of signal transduction pathways by Monocyte Chemotactic Proteins-1, -2, -3 and –4 in the THP-1 monocytic cell line: *Regulation of CCR2 signalling cascades by phosphatidylinositol –3-kinases.*

Submitted by

MARISA KAY LOGAN

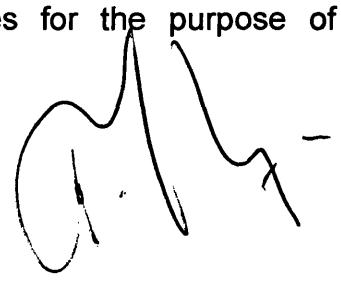
For the degree of Ph.D
of the University of Bath

2002

Copyright

Attention is draw to the fact that copyright of this thesis rests with its author. This copy of the thesis has been supplied on the condition that anyone who consults it is understood to recognise that its copyright rests with its author and that no quotation from the thesis and no information derived from it may be published without the prior written consent of the author.

This thesis may be made available for consultation within the University library and may be photocopied or lent to other libraries for the purpose of consultation.



UMI Number: U601983

All rights reserved

INFORMATION TO ALL USERS
The quality of this reproduction is dependent upon the quality of the copy submitted.

In the unlikely event that the author did not send a complete manuscript
and there are missing pages, these will be noted. Also, if material had to be removed,
a note will indicate the deletion.

UMI U601983
Published by ProQuest LLC 2013. Copyright in the Dissertation held by the Author.
Microform Edition © ProQuest LLC.
All rights reserved. This work is protected against
unauthorized copying under Title 17, United States Code.

ProQuest LLC
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106-1346

UNIVERSITY OF BATH
LIBRARY
40 20 NOV 2002
Ph.D.

TABLE OF CONTENTS

List of Figures

List of Tables

ABSTRACT

Monocyte chemotactic peptide-1 (MCP-1) is a member of the CC chemokine family, and is a potent inducer of monocyte and $CD45RO^+$ lymphocyte chemotaxis. *In vivo* studies suggest that MCP-1 recruits monocytes to sites of inflammation in a number of pathological conditions including atherosclerosis and rheumatoid arthritis. The cellular effects of MCP-1 are mediated primarily by binding to the CC chemokine receptor-2 and are reported to activate a number of signalling pathways. CCR2 is activated by multiple agonists, including MCP-1, -2, -3 and -4, however, to date the signal transduction events elicited by MCP-2, -3 and -4 remain unclear.

This thesis proposed to explore the signal transduction events elicited via CCR2. One of the aims was to compare the effects of MCP-2, -3 and -4 to those observed with the prototypical CCR2 ligand, MCP-1. To pursue these aims, the monocytic cell line, THP-1, was used as an experimental model for molecular signalling and functional studies alongside some supportive work being performed in the Jurkat cell line.

MCP-1, -2, 3 and -4 activated at least one member of class I PI3-kinase family in THP-1 cells. In addition, MCP-1 was shown to activate two distinct isoforms of the class II PI-kinases, PI-3K-C2α and PI3K-C2β. The ramifications of PI3K activation on downstream signalling events were also made apparent. CCR2-mediated activation of the downstream effectors of PI3K (namely PKB and GSK-3α/β) were observed, as well as PLD, and MAPKs - all of which were at least partially dependent on PI3K

The use of Clostridial toxins permitted the examination of the role of small GTP binding proteins (e.g. Ras) in PI3K regulation, and the data is indicative that these proteins function upstream of PI3K in this system. It is evident that small GTP binding proteins have a potential importance as signalling components involved in modulating efficient CCR2 coupling to downstream pathways and subsequent functional output.

MCP-1, -2,-3 and -4 activated three distinct major MAP kinases with a similar signalling profiles, albeit with slightly different kinetics. The only inter-chemokine differences observed with regards to MAP kinase activation were those pertaining to PKC-dependency. Activation of ERK-1/2, p38 and JNK were observed in response to all four ligands although exhibited different levels of dependency on PI3K. Differential regulation of these MAPKs by G-proteins was also observed in this system.

With regards to the functional readout in this system, it was demonstrated that MCPs-1-4 were capable of inducing chemotaxis, and as expected all four chemokines exhibited different degrees of potency. In each case, chemotaxis appeared to be a $G\alpha_i$-mediated event that was dependent on PI3K, but independent of ERK-1/2 activation.
This thesis highlights the sophisticated mechanisms underlying CCR2-mediated signal transduction, and the subsequent chemotactic response. In particular, it conveys the important role of PI3Ks in cellular function and its influence on other signalling pathways. This study has brought us a step closer to characterising the effect of PI3K and other signalling molecules on events stimulated by CCR2 ligation, and identifying the factors that lead chemokine-mediated inflammatory disease states, such as atherosclerosis. Identification of the cellular response to inflammatory stimuli may, in time, lead us to suitable targets for therapeutic intervention.

Abbreviations

A List of abbreviations commonly used within the text of this thesis. For a comprehensive review of chemokine acronyms, see Table 1.1, and for reagent abbreviations see Section 2A

7-TMS, seven-transmembrane-spanning; **Apo**, apolipoprotein, **ARF**, ADP-ribosylation factor; **ATP**; adenosine trisphosphate BPI, bactericidal permeability increasing factor; **BSA**, bovine serum albumen; **Btk**, Brutons tyrosine kinase; **CT**, cholera toxin; **DAG**, diacylglycerol: **DNA**; deoxyribonucleic acid; **GAG**, glycosaminoglycans; **GAP**, GTPase activating proteins; **GDP** guanine disphosphate; ;**PTX**, pertussis toxin; **GEF**, guanine nucleotide exchange factor; **GFP**, green fluorescent protein; **GPCR**, G protein-couple receptor; **GSK-3**, glycogen-synthase kinase; **GST**, glutathione S-transferase; **GTP**; guanine tripshosphate **HIV**, human immunodeficiency virus; **HPLC**, high performance liquid chromatography; **IL-8/13**, interleukin-8/13; $InsP_3$, inositol trisphoshate; **JAK**, janus-activated kinase; **JNK** c-Jun amino terminal kinase; **LDL**, low density lipoprotein; **LPS**, lipopolysaccharide; **LT**, lethal toxin; **MAb**, monoclonal antibody; **MAPK**, mitogen-activated protein kinase; **MEK**, **MAPK/ERK** kinase; **P70S6K**, P70S6-ribosomal kinase; **PA**, phosphatidic acid; **PAGE**, polyacrylamide electrophoresis; **PC**, phosphatidylcholine; **PDK**, phospoinositide-specific kinase; **PH**, plecktrin homology; **PI3K**, phosphatidylinositol 3-kinase; **PKB**, protein kinase B; **PLA/C/D**, phospholipase A/C/D; **PTB**, phosphotyrosyl binding; **PtdIns**, phosphatidylinositol; **PtdIns(3)P**, phosphatidylinositol 3-monophosphate; **PtdIns(3,4)P_2**, phosphatidylinositol (3,4)bisphosphate; **PtdIns(3,4,5)P_3**, phosphatidylinositol (3,4,5)trisphosphate; **PTEN**, phosphatase and tensin homologue deleted on chromosome ten; **PX**, phox domain; **RBD**, Ras binding domain; **RGS**, regulators of G protein signalling; **SAPK**, stress activated protein kinase; **SCF**, stem cell factor; **SH**, Src homology; **SHIP**, SH2-domain-containing inositol polyphosphate 5-phosphatase; **SIV**, simian immunodeficiency virus; **TLC**, thin-layer chromatography.

ACKNOWLEDGMENTS

I would like to express my appreciation for Dr Stephen Ward – An attentive and loyal supervisor who has guided me through the past four years. Furthermore, he has shown that this support continues even as my career path takes me further away from research. Invaluable assistance has also come from our industrial collaborations with Glaxo-Smithkline Pharmaceuticals. It is fair to say that this project would not have been as fruitful without the financial and scientific contribution from Dr Theo Berkhout. With GSK, Theo has been a positive influence on this study, has shown a keen interest in our group as a whole, and has always done his best to honour our endless shopping lists.

Thanks must also go to.... The unsung heroes of 7 West, namely Dr Adam Curnock and Mr Kevin Smith, who continually help and advise other researchers - frequently at the expense of their own agenda.....The Ward group and Dr Sean Weaver.....Dr Welham and her postdocs...postgraduate office dwellers (old and new)....Miss Laura McAuliffe – a good mate and fellow scientist-cum-civil servant.....and Mrs Sandie Broom who has been my surrogate mother during the writing of this thesis.

And very special 'thank-you's' to.....

Dr Karen Wright – For providing friendship and support above and beyond the call of duty.....Mr Steven 'Budgie' Burgess - who has been a great friend and co-~~shirker~~ worker throughout this experience, and for whom I have tremendous respect for his strength and determination throughout 2001.....Timothy Newstead Phillips - who has endured so much over the past two years (Thank-you T for helping me to keep it together, and for teaching me about the other important things in life. X)

Finally, this thesis is dedicated to Mum and Dad Logan. Two fine parents who have aided me financially and emotionally for all my years and have worked incredibly hard to give Luke and I the chance to obtain a good education. Their way of providing opportunity and encouragement yet without pressure or expectation has been something that I have always been very grateful for. Furthermore, they continued to have faith in me and maintained their support even during the 'party years'.
I am blessed.

Dedicated to Mrs Evelyn Grace

"There is at best only one cure for disease. Phagocytes! Stimulate the phagocytes, drugs are a delusion."

George Bernard Shaw. The Doctors Dilemma. (1913)

Section I: INTRODUCTION

1.1 Inflammation

Inflammation is essentially a protective response intended to eliminate both the initial causal agents (e.g. microorganisms, toxins) and clear necrotic cells and tissues that arise as a consequence of such injury. Inflammation is classified into two basic forms; (1) *Acute* inflammation, which is of relatively short duration (lasting from a few minutes up to a few days) and characterised by a predominantly neutrophillic leukocyte accumulation. (2) *Chronic* inflammation, which is of longer duration (days to years) and characterised by the influx of lymphocytes, monocytes and by ongoing tissue destruction and repair.

Acute inflammation is the immediate response to injury. The critical function of this early response is to deliver leukocytes to the site of inflammation, to counteract infectious agents as well as degrade necrotic tissues. Conversely, leukocytes themselves may also prolong inflammation, and induce damage in their release of oxygen free radicals, enzymes and inflammatory mediators (Tsokos *et al,* 1995; Kumar *et al*, 1997).

In contrast to acute inflammation which is distinguished by vascular changes (vasodilation), and a largely neutrophillic infiltrate, chronic inflammation is characterised by (1) infiltration with mononuclear cells, including macrophages, lymphocytes and plasma cells, (2) destruction of tissue, and (3) tissue repair involving proliferation of vessel wall (angiogenesis) and fibrosis.

Chronic inflammation may follow acute inflammation, and such a transition may arise when the acute response cannot be resolved, either because of a persistent infectious agent (e.g. infection) or because of interference in the normal process of healing (e.g. prolonged exposure to toxic agent).

"Chronic Inflammatory Cells"

Macrophages are a principle component of the *mononuclear phagocyte system*, which consists of closely related cells of bone marrow origin, and their accumulation is a characteristic feature of chronic inflammation. Under the influence of adhesion molecules and chemotactic factors (e.g. MCP-1, MIP-1α), circulating *monocytes* begin to immigrate to the site of injury. When monocytes reach extravascular tissue,

they undergo transformation into larger, phagocytic cells called *macrophages.* Macrophages themselves may also become activated upon several inflammatory signals including bacterial toxins, extracellular matrix proteins (e.g. fibronectin) and cytokines secreted by T lymphocytes (in particular INF-γ). In short, macrophages have three major roles in resistance to infection: Firstly, they destroy antigen by phagocytosis. Second, they process the antigen for immune response and serve to regulate this response through Interleukin-2 production, thus promoting T cell proliferation and; Thirdly, they are potent effectors of the inflammatory response by their capacity to release arachidonic acid-derived mediators, cytokines and respiratory burst products (Petit and Hoffbrand, 1992).

Introduction of the appropriate leukocytes into close proximity with the offending infectious agent is a highly co-ordinate process involving the integration of many inflammatory molecules. The sequence of events in the extravasation of leukocytes from the vascular lumen to the extravascular space is a multistep process, collectively known as *cell migration* or *chemotaxis.*

Cell Migration

On a cellular level it is now accepted that leukocyte migration is a multi-step process that involves sequential interactions with several sets of adhesion molecules on the leukocyte that interact with corresponding receptors on the endothelium (For review, see Springer 1994). The initial step of this process is the slowing and 'rolling' of leukocytes on the endothelium and which is mediated by 'selectins'. Selectins are characterised by an extracellular domain that binds sugars (hence the "lectin" part of the name). These include E-selectin, exclusive to endothelium; P-selectin, present on endothelium and platelets; and L-selectin, found on the surface of most leukocytes. (Tedder, 1995; Butcher and Picker, 1996) Since a leukocyte spends only seconds in a venule, it is imperative that becomes rapidly tethered. Selectins have the ability to bind their ligands in milliseconds, thus, providing a crucial mechanism whereby the leukocyte is quickly anchored down, and suitably positioned for the next stage to take place.

The second step is mediated by via chemotactic agents and their induction of 'integrin' adhesiveness. Rolling is ceased and firm integrin-mediated adhesion takes place. Integrins are a large family of molecules involved in intracellular and cell-substratum adhesion and can be characterised on the basis of their two-subunit

composition (Oppenheim *et al*, 1991; Baggiolini, 1998). The engagement of the integrin with their cognate receptors on endothelium is the third step in this transmigrational process. Here, the interacting cells are linked together in close proximity that allows development of the traction that is associated with migration. The adhesion is largely mediated by endothelial adhesion molecules which include ICAM-1 (intercellular adhesion molecule 1) and V-CAM-1 (vascular adhesion molecule 1), both of which have increased surface expression following stimulation by various inflammatory mediators. Integrins are transmembrane heterodimeric glycoproteins that also function as cell receptors for the extracellular matrix (ECM); the principal integrin receptors for ICAM-1 are LFA-1 (CD11a/CD18) and CD11b/CD18, while V-CAM-1 binds to the integrin VLA-4. These proteins are normally expressed on leukocytes, but do not adhere to their appropriate ligands until the leukocytes are activated – only at this point do they undergo the conformational change necessary to confer ligation to the endothelial adhesion molecules (for reviews; Gahmber *et al*, 1999; Aplin *et al*, 1999).

The process of slowing down the leukocyte has commonly been described as transient 'arrest", and is the initial step in active cellular migration. Transendothelial migration, or 'diapedesis' involves the movement of the leukocyte through the endothelial cell wall and subsequent interstitial movement towards the chemoattractant/chemokine. This is a highly orchestrated process that requires the sequential co-ordination of the following events:

i) Polarisation of the cell by formation of a 'leading edge' and a 'uropod'. Formed from the redistribution of signalling molecules, cytoskeleton, chemokine receptors and cell surface molecules. Probably directing cells along a chemotactic gradient.

ii) Protrusion of membrane extensions such as filopodia and lamellipodia. A reversible event that is governed by reorganisation of local F-/G-actin.

iii) Establishment and maintenance of cellular attachment co-ordinated by the synthesis and redistribution of activated integrin molecules (e.g. ICAM-1, ICAM-3, and CD44) to the leading edge.

iv) Contraction of cellular body and adhesive traction. A process regulated by myosin-based ATP-motors and integrin binding.

v) Release and retraction of the uropod - a mechanism thought to be under the control of integrin endocytosis.

(Lauffenburger *et al*, 1996; Nieto *et al*, 1997; Sanchez-Madrid *et al*, 1999)

After extravasation, leukocytes emigrate toward the site of injury along a chemical gradient, in a process referred to as 'chemotaxis'. Both exogenous and endogenous substances can act as chemotactic agents for leukocytes, including: soluble bacterial products, components of the complement system (e.g. C5a), products of the arachidonic metabolism pathway (e.g. leukotriene B_4) and cytokines, particular those of the chemokine family (e.g. IL-8). This process is shown schematically in Figure 1.1.

Figure 1.1

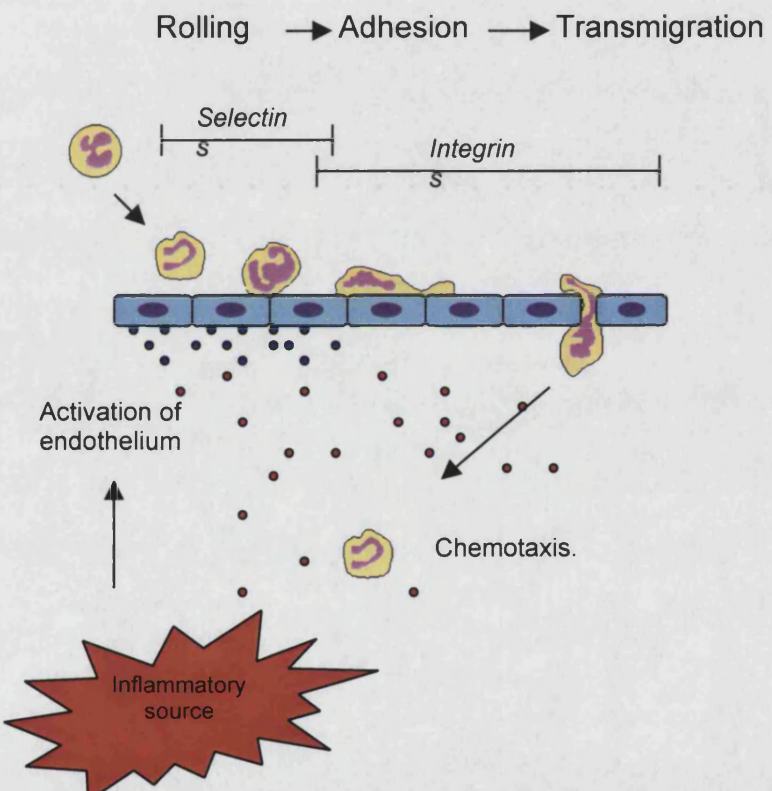


Figure 1.1 Sequence of events in leukocyte emigration in inflammation (simplified). The leukocytes (1) roll, (2) arrest and adhere to endothelium, (3) transmigrate through intracellular junction (extravasation), (4) migrate towards chemoattractant.

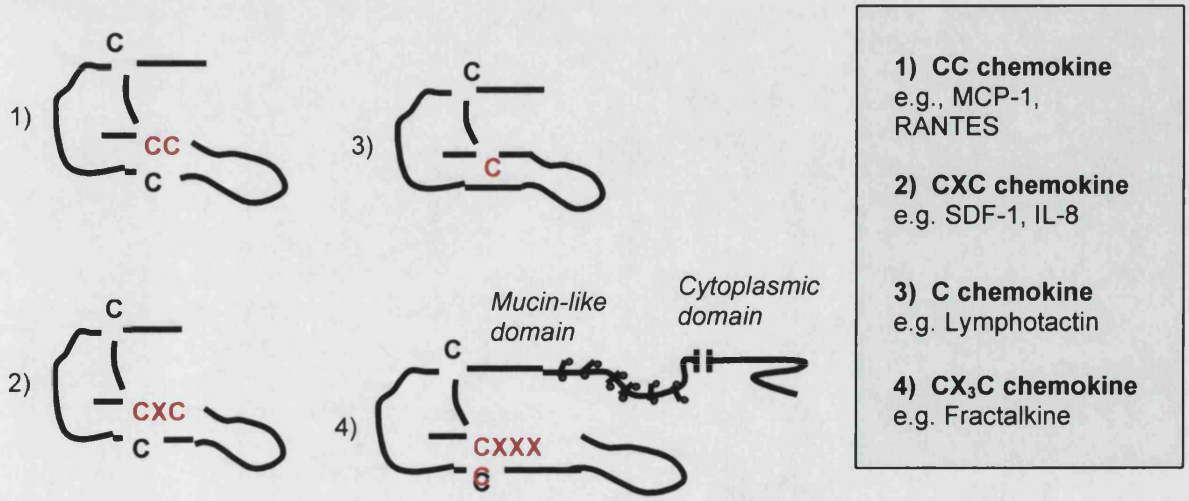
In the case of the phagocytic chemokines, receptor-mediated interaction is followed by the engulfment of the foreign agent in a phagocytic process. Once internalised by the ingesting leukocyte, a 'respiratory burst' is induced. This is characterised by a rapid, marked increase in the consumption of oxygen by the cell, resulting in the production of toxic metabolites, such as superoxide, hydrogen peroxide, hydroxyl radicals and myeloperoxidase (Beron *et al*, 1995). It is important to note that even in the absence of an oxidative burst, other constituents of leukocyte granules are capable of killing bacteria or infectious agents. These include lysozyme, lactoferrin, defensins and bactericidal permeability increasing protein (BPI).

1.2 Chemokines

The Chemokine Superfamily

Chemokines are a rapidly growing superfamily of proinflammatory cytokines that serve to attract and activate specific types of leukocytes (For reviews see Baggiolini, 1997; Hendrick, 1997; Thelen, 2001). Over 13 years ago, the first chemokine, now known as CXC chemokine ligand 8 (CXCL8), formerly known as interleukin-8 (IL-8) was identified as a neutrophil attracting factor, and opened the doors to a new concept in chemotactic agents. In recent years, these chemotactic cytokines, which are in the 8- to 17-kDa molecular mass range, have been shown to selectively attract certain subsets of leukocyte populations to sites of inflammation. This superfamily has been divided into 4 groups (CXC, CX_3C, CC and C) pertaining to the position of the first two N-terminal cysteines of the amino acid sequence (*Figure 1.2*).

Figure 1.2 Schematic Representation of chemokine Families



The CXC (or α) family has the first two NH_2-terminal cysteines separated by one non-conserved amino acid residue. The CXC chemokines can be divided into two subclasses based on the presence or absence of an ELR amino acid motif positioned immediately upstream of the first N-terminal cysteine. The presence or absence of this motif has been associated with distinct and opposite functional effects. For example, ELR+ CXC-chemokines are quite often angiogenic whereas lack of ELR renders these chemokines angiostatic (Schall and Bacon, 1994).

In contrast, the CC (or β) family holds these cysteines in juxtaposition, the C (γ) family has just a single NH_2-terminal residue, while the CX_3C (or δ) family has three intervening amino acids separating these cysteine residues. Most of the chemokines fall into the CXC or CC families since there is only one C (lymphotactin) and one CX_3C chemokine (fractalkine) identified in humans.

'Fractalkine', the only member of the CX_3C is a notable exception to other soluble chemokines in that it is an integral membrane protein, heavily substituted with mucin-like carbohydrates and a chemokine domain at its N-terminus. Such a structure supports the view that soluble secreted chemokines bind to, and are immobilised by cell surface heparan sulphates or other glycosaminoglycans (GAGs). It is thought that the immobilisation of chemokines by GAGs forms stable, solid-phase chemokine foci and gradients required for efficient trafficking (Witt and Lander, 1994). Consistent with this proposal, immobilised IL-8 and RANTES have been shown to promote leukocyte migration and adhesion, respectively (Middleton *et al,* 1997). Binding of chemokines to GAGs has also been shown to induce multimerisation, increase their effective local concentration and optimise receptor occupancy (Hoogwerf *et al*, 1997). A clearer understanding of chemokine-GAG interactions may be useful in explaining functional differences between chemokines that according to other criteria, have been judged as functionally redundant.

The other exception to the rule is the C chemokine 'lymphotactin' and is so far the only member of this putative family. Although it is the correct size, and shows characteristic sequences common to CC chemokines, it has a lone cysteine at the N-terminal domain. Nevertheless, it is upregulated in several inflammatory conditions and is a potent attractant for T lymphocytes (Murdoch and Finn, 2000).

During the last 5 years, the development of EST databases has been primarily responsible for the identification of many new chemokines and their receptors. Chemokines represent ideal candidates for discovery through bioinformatics because

of their relatively small size and highly conserved motifs, and this has resulted in the rapid identification of new chemokines. Unfortunately, this has also led to severe trouble with chemokine nomenclature, as multiple names have been published for the same molecule. Recently, the 'definitive' update for chemokine classification was proposed by the Nomenclature Committee of the International Union of Pharmacology XXII and the various names and acronyms were reassigned accordingly (Murphy *et al*, 2000). The CC chemokines, were renamed 'CCL' (CXCL1-27) , and 'CXCL' was reassigned to all the CXC chemokines (CXCL1-14). In spite of the revised nomenclature, chemokines continue to be referred to by their classical names, so to avoid confusion, all classical names shall be used from hereon in. The complete revised list of identified chemokines to date (showing revised and classical names) is shown in Table 1.1.

Table 1.1 Chemokines and their Receptors

CHEMOKINE (classical/systematic name)		**RECEPTOR**	**TARGET CELLS**	**MAIN EFFECT**
CXC (ELR+)				
IL-8/CXCL8	CXCL8	CXCR1	N.B.T	Degranulation
GROα/CXCL1	CXCL1	CXCR2	N	Endo. proliferation
GROβ/CXCL2	CXCL2			Tumour enlargement
GROγ/CXCL3	CXCL3			Liver regeneration
ENA-78	CXCL5			
LDGF-PBP/			F	
GCP-2	CXCL6		N	
CXC (ELR-)				
PF4	CXCL4	CXCR3	F	Angiostasis
MIG	CXCL9		T* (Th1>Th2)	Angiostasis, antiproliferative, anti-tumour,
IP-10	CXCL10			
ITAC	CXCL11			
SDF-1α/β	CXCL4	CXCR4	Ubq. T, DC, BM	Embryonic GI, CNS. B. Cell dev. Antagonism of tHIV-1 Entry.
BCA-1	CXCL13	CXCR5	B	B cell development

Table1.1 Chemokines and their receptors (continued)

CC (β)				
MIP-1α	CCL3	CCR1, CCR5, CCR9	M Ma. NK. Bas. DC. BM. B. T	NK activation. IgE/IgG prod. mHIV entry antagonism. T costim.
MIP-1β	CCL4			
MDC	CCL22	CCR4	DC. T. (Th1>Th2)	Intra-thymic Dev. Trafficking. +/- ive selection. Th2 responses
TECK	CCL25	CCR9/11	Ma. Thy. DC.	
TARC	CCL17	CCR4/8	T cell lines	
RANTES	CCL5	CCR1/CCR3/CCR5	M. Ma. NK. DC. T^M. T. Bas.	T cell prolif. mHIV-1 entry antagonism
DC-CK-1	CCL18	CCR1	T^N > T	Promotes T :DC antigen pres.
HCC-1	CCL14	CCR1/CCR9	M.	Anti-proliferative for progenitor BM.
HCC-2	CCL15	CCR1/CCR3		
HCC-4	CCL16	CCR1/CCR8	M. Lym.	
MIP-3α	CCL20	CCR6	PBMC. BM. T^M > T	
MIP-3β	CCL19	CCR7	T^N > T	T cell homing
MCP-1	CCL2	CCR2, D6	T. M. Bas.	NK, T, Bas degran. Mon/mac activation
MCP-2	CCL8	CCR1, CCR2, CCR3, CCR5 ? D6	T. M. Bas. Eo.	
MCP-3	CCL7	CCR1/CCR2/CCR3/	T. M. Bas. Eo. DC	
MCP-4	CCL13	CCR2/CCR3	T. M. Bas. Eo. DC	Eo,Bas degran.
Eotaxin	CCL11	CCR3	Eo.	Eo activation
Eotaxin–2	CCL24	CCR3	Eo. Th2. Bas	
I-309	CCL1	CCR8	T	Th2 responses?
MIP-5		CCR1/CCR3	T. M. N.	?
MPIF-1	CCL23	CCR1	M. T^R	Anti-proliferative for myeloid cells
6C-kine	CCL21	CCR7/CCR11	T. B. Mes.	Anti-proliferative for BM progenitor
C(γ)				
Lymphotactin	XCL1		T. NK.	Anti-tumour/viral.
CX_3C (δ)				
Fractalkine	CX3C L1		Nero. Endo. NK. T (CD8)	Neurological inflamm. responses
Promiscuous				
Multiple CC and CXC (ERL+) chemokines	DARC		RBC.	Coreceptor for Malarial parasite.

Legend for Table 1.1. **Chemokines:** IL-8, Interleukin-8; GRO-α/β/γ, Growth related oncogene-α/β/γ; ENA-78, Epithelial derived attractant-78, LDGF-PBP, Leukocyte derived growth factor-platelet basic protein; GCP-2, Granulocyte chemotactic protein-2; PF4, Platelet factor-4; MIG, Monokine induced by Interferon-gamma; IP-10, Interferon-γ inducible protein-10; ITAC, Interferon-inducible T cell alpha chemoattractant; SDF-α/β, Stromal-cell derived factor-α/β; BCA-1, B cell attracting chemokine-1; MIP-1 and –3 α/β, Macrophage Inflammatory protein-1 and –3 α/β; MDC, Monocyte derived chemokine; TECK, Thymus-expressed chemokine; TARC, Thymus and activation-regulated chemokine; RANTES, Regulated on activation normal T cell expressed and secreted; DC-CK-1 (PARC), Dendritic cell chemokine-1 (Pulmonary and activation-regulated chemokine); HCC-1,2,4, Human CC cytokine 1,2,4; LARC, Liver and activation-regulated chemokine; ELC, EBI1-ligand chemokine (EBI1+CCR7), MIP-3 αβ and MIP-5 (See MIPα/β); MCP-1, -2, -3, -4, Monocyte chemotactic protein-1,2-3,-4; MPIF-1, Myeloid progenitor inhibitory factor-1; 6C-kine/SLC, Six-cysteine chemokine/Secondary lymphoid tissue chemokine. **Cells:** N, Neutrophils; B, B cells; T, T cells; T*, activated T cells ; T^M, Memory T cells ; T^N, Naive T cells, T^R, Resting T cells ; Th1/2, T helper cell type 1 & 2; Thy, Thymocytes; Bas, Basophils; Mes, Mesangial cells; RBC, Red blood cells; PBMC, Peripheral blood mononuclear cells; Endo, Endothelial cells; NK, Natural killer cells ; Ubq, Ubiquitously expressed; DC, Dendritic cells; Lym, Lymphocytes.

The Functional Role of Chemokines

The capacity of chemokines to both activate and attract various subpopulations of leukocytes has placed these molecules as key modulators in a vast array of biological processes. From early cellular development, its maturation and homeostasis, right through to ultimate cell death, it is becoming increasing clear that chemokines play an intrical part in each process. Fifteen years of research has pinpointed these proteins to organogenesis, embryogenesis and development (Wilkinson *et al*, 1999) angiogenesis (Voest *et al*, 1995) as well as both positive and negative regulators of apoptosis (Cerdan *et al*, 2001). Chemokines and their receptors have now been heavily implicated in various aspects of T-lymphocyte biology, in a way which is quite distinct from the established role as chemotactic agents for the movement of leukocytes in inflammation (for Reviews see Ward and Westwick, 1998, Ward *et al*, 1998). For example, the effects of chemokines on T cell function(s) have been shown in thymic development (Mebius *et al*, 1996; Campbell *et al*, 1999), lymphoid trafficking and differentiation of the Th1/Th2 subsets (Siveke *et al*, 1998; Sebastiani *et al*, 2001). The acknowledgement that many of these T cell responses can be attributable to chemokines is crucial to the search for

novel therapeutic strategies for immune deficiencies, autoimmune diseases and transplantation.

Transgenic and Knockout Mice: Models of Chemokine Action

The targeted disruption of chemokine and chemokine receptor genes as well as the expression of chemokines as transgenes has identified important roles for these molecules in specific aspects of the inflammatory response. The ability of chemokines to direct leukocyte infiltration depends on a low regional expression level. In studies with MCP-1 transgenic mice, the systemic overexpression of a mouse mammary tumor virus long terminal repeat-MCP-1 transgene led to a general paralysis of the response to this chemokine (Grewal *et* al, 1997). As well as persistent expression of MCP-1 expression and insulitis, the macrophage infiltrate did not result in progressive tissue destruction. Therefore, additional costimulatory signals are required for the activation of macrophages.

Knockout mice with a targeted disruption for either MCP-1 or its cognate receptor (CCR2) gene show a reduced capacity to recruit monocytes and have suggested a fundamental role for these molecules in the generation of atherosclerotic lesions (Boring *et al*, 1998; Lu *et al*, 1998). Mice deficient for CCR5 show an increased humoral response to T cell-dependent antigenic challenge, suggesting a novel role for CCR5 in downmodulating T cell-dependent immune responses (Zhou *et al*, 1999). Targeted disruption of the eotaxin gene demonstrated that eotaxin enhances the magnitude of early but not late eosinophil recruitment after antigen challenge (Rothenberg *et al*, 1997). CXCR4 is broadly expressed by cells of the immune and central nervous system and mediates the migration of resting leukocytes and hematopoietic progenitors in response to its ligand SDF-1 (Bleul *et al*, 1996) Essentially identical lethal defects were seen in mice deficient for either CXCR4 or SDF-1. These include severely reduced B lymphopoiesis, reduced myelopoiesis in bone marrow, defective formation of the large vessels supplying the gastrointestinal tract, as well as cardiac defects and abnormal cerebellar development (Zou *et al*, 1998; Ma *et al*, 1998;Tachibana *et al*, 1998). In normal mice, CXCR5 is expressed on mature B cells and a subpopulation of T helper cells. CXCR5 knockout mice lack inguinal lymph nodes and possessed little or no normal Peyer's patches. Lymphocyte migration into splenic follicles of these mice were significantly impaired (Legler *et al*, 1998).

Chemokines in the pathogenesis of disease.

Chemokines and their receptors have been extensively implicated in several disease states including arthritis, multiple sclerosis, pneumonia (for review see Lukacas and Kunkel, 1998; Gerard and Rollins, 2001), psoriasis (Schroder *et* al, 1996), atherosclerosis (Boring *et* al, 1998; Gosling *et al* 1999) cancer and tumour progression (Schneider *et* al, 2001). Chemokine receptors themselves have been targeted as vehicles of cellular invasion by a wide variety of microorganisms. These range from the binding of malarial parasite *Plasmodium vivax*, to the Duffy blood group antigen (a promiscuous chemokine receptor on erythrocytes), to the platelet activating factor receptor (PAFR), which is a window of entry for *Streptococcus pneumoniae.* However, the most impressive use of cellular receptor entry by a pathogen has to be that of the human immunodeficiency virus type 1 (HIV-1).

An exciting series of discoveries was the finding that certain chemokine receptors act as coreceptors for the HIV-1 virus, and this was based on the observation that a number of chemokines, such as RANTES and SDF-1 can suppress the infection of T cells with M tropic and T tropic HIV-1 strains, respectively (Cocchi *et al*, 1995; Bleul *et* al, 1996; Lu *et al*, 1997). Attractive as it may be to hypothesise that inappropriate activation of the chemokine network is responsible for disease pathology, the only human disease for which there is indisputable evidence for an association with chemokine expression is HIV. In all other cases, roles for chemokines have been largely inferred from animal models. Figure 1.3 shows the five major categories of disease in which animal models show a role for chemokines and for which correlative data exist in humans.

Monocyte chemoattractant protein-1

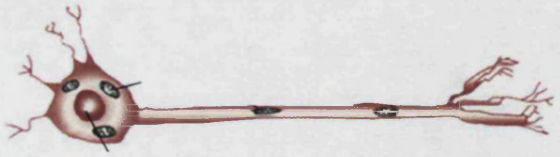
Monocyte chemoattractant protein-1 (MCP-1) is a member of the CC subfamily of chemokines and serves to attract an activate monocytes both *in vitro* and *in vivo* (Oppenheim *et al,* 1991). The gene was originally identified from its mouse homologue, *JE*, cloned from PDGF-stimulated mouse 3T3 cells, and was the first PDGF-inducible gene to be described (Ping *et al*, 1996). The human homologue, gene name "small inducible cytokine A2" [SCYA2], encodes MCP-1 and this is structurally related to the other members of the MCP cytokine family. MCP-1 has

Autoimmune Disease

Rheumatoid Arthritis
Increased levels of MCP-1 and MIP-1α found in synovial tissue. Anti-MCP-1 and RANTES ameliorated AA in rats. DNA vaccines to CC chemokines inhibit progression of AA (Yousef *et al*, 2000)
Models: Autoimmune Arthritis (AA)
Multiple Sclerosis. High levels of RANTES, MIP-1α, and MCP-1 in inflammatory lesions. IP-10 and MIG recruit T cells to lesion. CCR5 not necessary for development, but implicated in disease progression (Sorensen *et al*, 1999). MCP-1 and CCR2 viewed as crucial potential therapeutic targets (Simpson *et al*, 2000).
Models: Experimental allergic encephalitis (EAE). Humans.

Infection

HIV. Evidence that individuals deficient in CCR5 cell surface expression (due to homozygous carriage of Δ32 deletion) conveys protection towards HIV virus.
CXCR4 shown to be coreceptor for T-tropic strains of HIV (Lu *et al*, 1997).
AIDS: Although likely to be multifactoral, AIDS pathogenesis is thought to involve chemokine receptor expression. General broadening of viral tropism and variable degree of usage of additional chemokine receptors. Promotes virulence?
CCR5 and CCR3 implicated in AIDS dementia. (Littman, 1998)
Models: SIV, Human.

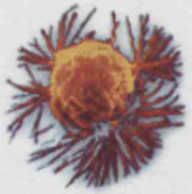


Transplantation

Allograft. Heart and skin transplants. Neutrophil and monocyte-active chemokines, such as MIP-2, KC and MCP-1, respectively, appear around graft wounds. Alteration in chemokine profile develops several days after graft: Persistence of MCP-1 expression with CXCR3 ligands (IP-10, MIG, I-TAC), appearing along side CCR5 ligands (MIP-1β and RANTES).
Mice deficient in CCR2, CCR5 and CXCR4 all demonstrated an improved survival rate to grafts. Most impressive response to allografts was shown with CXCR3 knockout mice (Hancock *et al*, 2000).
Models: Heart allograft, sponge allografts

Inflammation and Allergy

Asthma
Th2 cells have significant role in atopy and allergic disease. Correlation of this Th2 has been observed with expression of CCR4 and CCR8, and therefore makes these receptors potential candidates for therapy. (D'Ambrosio *et al*, 1998)
In asthmatics, there is significant upregulation of eotaxin in bronchial mucosa, and CCR3 expression on infiltrating eosinophils. Th2 cytokines (esp. IL-13) induce eotaxin, MCP-1 and MIP-1α in the lung. Some CC chemokines cause degranulation of basophils, thus exacerbating local inflammation.
Chemokine neutralisation studies and gene targeting only partially reduce recruitment - due to redundant action of leukocyte-attracting chemokines in the lung. (Gonzalo *et al*, 1998)
Models: Human, Rat asthma models.



Neoplasia.

Chemokines recruit tumour-associated leukocytes, in particular MCP-1. Contribution to tumour angiogenesis in breast cancers through macrophage infiltration (MCP-1). CC and CXC chemokines and receptors expressed by many tumour cells.
IL-8 stimulates proliferation in lung cancers. (Gerard and Rollins, 2001)
Chemokines have a role in malignant transformation?
Models: Rat tumour model

Vascular.

MCP-1-CCR2 implicated in macrophage infiltration and foam cell accumulation in atherosclerosis MCP-$1^{-/-}$ mice have 65-85% less arterial deposition in hypercholesterolaemia models (Gosling *et al*, 1999). See text for further discussion.

Figure...The Role of Chemokines in Human Disease. Examples of disease states in which chemokines play a role in pathogenesis, including cardiovascular disease, allergic inflammatory disease, transplantation, neuroinflammation, cancer and infection.

been assigned to 17q11.2-12 (Rollins *et al,* 1991), the locus to which, MCP-2, -3 and 4 have since been mapped.

The monomeric structure of MCP-1 consists of an N-terminal loop and a β-sheet overlaid by an α-helix at the C-terminal tail (Lubkowski *et al*, 1997). Originally isolated from the culture supernatants of human malignant tumour cells lines, MCP-1 was thought to be a tumour-derived chemotactic factor for monocytes, but has since been shown to attract T and B lymphocytes, NK cells, and mast cells. As well as leukocytes, MCP-1 has been reported to attract and activate fibroblasts (Gharaee-Kermani *et al,* 1996), smooth muscle cells (Shecter *et al*, 1997), astrocytes (Hessen *et al*, 1996), and cells within the hepatic system, such as stellate cells (Marra et *al,* 1999).

MCP-1 mediates its cellular effects primarily through its binding to CCR2, which exists in A and B forms that arise via alternative splicing of the carboxyl-terminal tail. Engagement of the CCR2B splice variant, in particular, has been reported to stimulate a number of signalling pathways in primary cells and cultured cell models (Turner *et* al, 1998; Mellado *et* al, 2001). A wide variety of cells, including monocytes, fibroblasts, vascular endothelial and smooth muscle cells produce MCP-1 in response to a number of inflammatory mediators These include platelet-derived growth factor (PDGF), tumour necrosis factor-α (TNF-α) and lipopolysaccharide (LPS) (Ping *et al,* 1996; Valente *et al,* 1998). In addition, due to the growing evidence that MCP-1 plays a role in atherogenesis, two well-documented atherogenic stimuli such as oxidised low density lipoproteins (Berliner *et al*, 1990) and fluid sheer stress (Shyy *et al*, 1994) directly induce MCP-1 expression.
Induced expression of MCP-1 by these mediators is thought to be heavily dependent on transcriptional regulation by NF-κB. To date, several signalling pathways have been shown to mediate NFκB activation, recruitment of transcriptional complexes and the subsequent production of MCP-1 (Alsonso *et al*, 1999; Goeber *et al*, 2001).

MCP-1 has been considered as an important cytokine in mediating monocyte tissue infiltration in a variety of inflammatory diseases. These include atherosclerosis (discussed later), alveolitis, idiopathic pulmonary fibrosis, viral meningitis, glomerular nephritis, ovarian carcinoma and human breast cancer (Koch *et al* , 1992; Ransohoff *et al*, 1993; Luster, 1998; Ueno *et al*, 2000). Given the role of MCP-1 under pathophysiological conditions, therapeutic strategies directed at counteracting MCP-1

are flourishing within the pharmaceutical world. For example, anti-MCP-1 gene therapy and strategies employing antagonists of MCP-1 have already been shown to have comprehensive inhibition of vascular remodelling and tumour growth in atherosclerosis and cancer, respectively (Salcedo *et al*, 2000; Egashira *et al*, 2001),

MCP-2, MCP-3 and MCP-4

Following the characterisation of MCP-1, subsequent years brought about the expansion of the MCP family. To date, there are four human members of this chemokine family (MCP-1,-2,-3 and -4), all with similar structural and functional characteristics, and immune cell targets. As far as MCP-2, -3 and -4 are concerned, information regarding their exact function is just beginning to be elucidated. MCP-1 provides a suitable prototypical model for investigation into the role of other MCP family members in the immune response. (Van Damme, *et al*, 1992*)*. Sharing similar characteristics to that of MCP-3, MCP-2 recruits and activates a number of inflammatory cells, including monocytes, T lymphocytes, NK cells, basophils, mast cells and eosinophils, however, in contrast to MCP-3 it does not serve to activate eosinophils (Dahinden *et al*, 1994). Its ability to target a wide range of cells may be owed to it binding specificity and functional responses through receptors other than CCR2.

MCP-3 is a particularly interesting CC chemokine which was originally purified from osteosarcoma cell cultures, and was subsequently cloned in 1993 (Opendaker *et al* . 1993). Like MCP-2, it is a chemokine that is structurally and functionally related to MCP-1 (Sozzani et al, 1994), and the leukocyte receptors mediating MCP-3 responses are thought to be fully defined. However, it is one of the most broad acting chemokines, potently inducing chemotaxis of monocytes, basophils, eosinophils and lymphocytes, as well as degranulation of basophils eosinophils and monocytes (Combadiere *et al*, 1995).

In 1997, Berkhout *et al*, described the characterisation of a novel human CC chemokine and tentatively named it monocyte chemotactic peptide-4 (MCP-4). This chemokine showed a high level of sequence homology with human MCP-1. Using chemokine binding studies as well as functional response data, the receptors involved in MCP-4 binding and signalling have since been identified as CCR2b, CCR3 and CCR9/D6 (Stellato *et al,* 1997; Nibbs *et al*, 1997).). Like MCP-1, expression of MCP-4 has been demonstrated in human atherosclerotic lesions and is

known to induce monocyte chemotaxis in carotid and coronary arteries (Berkhout *et al*, 1997).

Table 1.2 The MCP chemokine family.

	Sequence Homology to other CC chemokines	Receptor	Target cell	Pathology
MCP-2	60% to MCP-1 and 3 30% to MIP-1α / RANTES	CCR1,CCR2 CCR3, CCR5,D6	M. T. NK. Bas. Ma. Eo.	Antagonist for CD4/CCR5 HIV entry (Gong *et al*, 1997)
MCP-3	71% MCP-1 25% RANTES 30% MIP-1α	CCR1, CCR2 CCR3, D6	M. T. Bas. Eo.	Multiple sclerosis (McManus *et al* 1998). Coreceptor for HIV (Blanpain, 1999). Irritable bowel disease (Helwig, 2000)
MCP-4	62% MCP-1	CCR2, CCR3,D6	M, T, B, Eo.	Atherosclerosis (Berkhout *et al*, 1997). Asthma (Taha *et al*, 2001) Allergic Rhinitis (Kaplan, 2001)

(See page 9 for legend)

1.3 Chemokine Receptors

In 1990, the cloning of human formyl-peptide receptor was a significant milestone in the efforts to characterise chemokine receptors. Through subsequent sequence analysis and the deduction of three-dimensional structures, high-resolution images of many other receptors are now being revealed. Today, 18 different chemokine receptors are known with over 30 chemokines characterised (See Table 1.1).

Figure 1.4. Structure of a generic CC chemokine receptor.

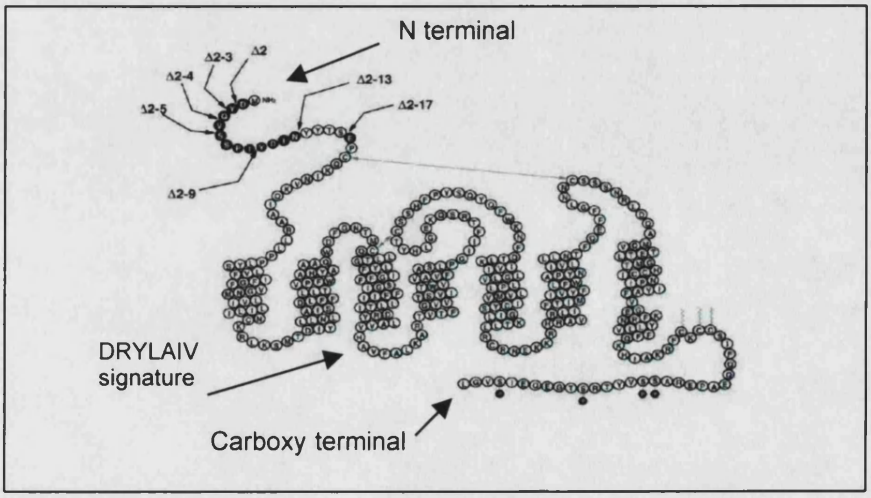


Figure 1.4. Amino acid composition o CCR5 is shown her to depict the typica structure of a C chemokine receptor

All of the receptors identified thus far are members of the seven-transmembrane spanning (7-TMS), G-protein-coupled (GPCR) family. Figure 1.4 shows a schematic representation of a generic chemokine receptor. Although showing similarities to many other 7-TMS receptors, chemokine receptors have exclusive structure signatures such as the DRYLAIV in the second intracellular loop. The major features of chemokine receptors are as outlined: They consist of approximately 350 amino acids; a short extracellular N-terminal tail (acidic) that contains glycosylation sites; an intracellular C-terminus which holds serine and threonine residues for signal regulation; seven α-helical transmembrane domains (3 intracellular and 3 extracellular connecting loops); lightly conserved cysteines between loops 1 and 2 connected by disulphide bridge (critical for general conformation of the molecule); G proteins coupled through C-terminus and possibly through the third intracellular loop (Murdoch and Finn, 2001). These receptors are, for the most part, able to couple to multiple G-protein isoforms, and it is this differential coupling to G proteins (and other downstream effectors) which confers a chemokines ability to elicit diverse physiological responses.

Chemokine receptor-coupling to G-proteins and 'Classical' Signalling Cascades.

GPCRs act as specific detectors for extracellular signals to be transduced into the stimulation of G proteins. Instead of directly acting on the effector proteins, the receptor uses G proteins as an interface to provide an extra step for signal amplification. Thus, these receptors can stimulate multiple G proteins that, in turn, regulate multiple effector proteins.

As for all 7TM receptors, ligand binding shifts the equilibrium between active and inactive receptor conformations in favour of the active one, with consequent exchange of GDP for GTP. Activation of the G protein by GTP not only dissociates the G-protein subunits, but also uncouples the G protein from the receptor, which renders the latter with little affinity for the agonist (For review see Hamm, 1998). G proteins are made up of α, β and γ subunits, with the α-subunits being the best characterised. Although there are multiple gene products encoding each α-subunit, only four main classes have been characterised to date: G_s, G_i, G_q, and $G_{12/13}$. See Table 1.3.

Table 1.3. G protein α subunits

Class	Members	Toxin sensitivity	Signal
G_s	α_s, α_{olf}	CT	↑cAMP ↑Ca^{2+}
G_i	α_i1,	PTX	↓cAMP
	α_i2, α_i3,	PTX	ΔVoltage
	α_o	-	↓Ca^{2+}
	α_t1, α_t2, α_{gust}		↓cGMP
	α_z		↓cGMP
G_q	α_q, α_{11}, α_{14}, α_{15}, α_{16}	-	↑IP_3, DAG
G_{12}	α_{12}, α_{13}	-	

Table 1.3. **G Protein sub-units**. Several of the α subunits can be modified by ADP ribosylation; pertussis toxin (PTX) and cholera toxin (CT). Abbr. AC, adenyl cyclase; cGMP-PDE, cGMP-dependent phosphodiesterase; PLC, phosphatidylinositol-specific phospholipase C; DAG, diacylglycerol.

Upon GTP binding to the α subunit, the αGTP and the βγ subunits dissociate, and in this new active conformation, the Gα subunit can interact with effectors with 20-100-fold higher affinity than in their GDP bound form (Neer *et al*, 1995). The four major classes of Gα subunits have well known cellular targets, and with the advent of the yeast two-hybrid screening technology, molecular studies have uncovered new targets. For example, GAIP, (a Gα-interacting protein and a member of the RGS family of GTPase-activating proteins) was identified by this technique, and more recently, nucleobindin was found as a putative target of α-subunits (Lin *et al*, 1998). GPCR-mediated signal transduction, however, is not exclusive to α-subunit activation. Let us now consider the role of Gβγ subunits:

Once Gα has dissociated from Gβγ, the liberated βγ is an activator of a multitude of proteins, and the list continues to increase (Clapham and Neer, 1997). The most clearly defined effects have been on classical second messengers such as phospholipase C-β_2, G-protein-responsive ion channels and; the Rho family of GTPases. Over the past 5 years, more interest has been generated around the βγ-mediated activation of PI 3-kinase isoforms, and Gβγ-stimulation of the MAP kinase pathway. It is quite apparent that these subunits are heavily influential on the control of both of these important signalling cascades. Given this rich and flourishing list of Gβγ effectors further investigation is needed to identify the pathways that ensue from βγ subunit release.

Chemokine Receptors and G proteins

There is considerable heterogeneity of chemokine receptors (and their isoforms) in their coupling to G protein subunits to initiate signalling pathways. Although it is considered that chemotaxis is dependent on coupling to the pertussis toxin sensitive $G\alpha_i$, it is becoming increasing clear that many chemokines demonstrate a limited heterogeneity (Kostensis *et al*, 1999).

In terms of migration, the $G\alpha_i$ subunit *per se* does not appear necessary. The essential step is the release of $\beta\gamma$ subunits from the G_i-coupled receptor. Only $\beta\gamma$-subunits released from G_i-coupled receptors have been shown to mediate chemotaxis, and not those from G_s or G_q. This has been further investigated by an elegant study by Neptune *et* al (1997), using receptors that do not bind chemokines or induce cell movement. These receptors (e.g. opioid) stimulated chemotaxis via G_i proteins when expressed on migration-competent cells. In contrast, neither G_s nor G_q-coupled receptors stimulated chemotaxis even when expressed in the same migration competent cells. These findings have been further supported by cellular distribution experiments using GFP-tagged $\beta\gamma$-subunits in *Discoideum* (Jin *et al*, 2000). Taken together these observations demonstrate that the release of $\beta\gamma$ is a crucial step in chemotaxis, but accompaniment by other signals generated by chemokine receptor activation is required.

The ability of chemokine receptors to couple to these multiple G-protein isoforms is, in many cases, facilitated by the DRYLAIVH motif in the second intracellular loop (Wu *et al*, 1993),. In addition, structural and mutational studies have implied that receptor-G-protein interactions are modulated by other intracellular domains including the second and third intracellular loops (Samson *et* al, 1997; Alkhatib *et al,* 1997; Zhou *et* al, 2001) and the cytoplasmic tail. Many strategies employing recombinant DNA techniques have been used to elucidate the structure-function relationship of chemokine receptors. Construction of hybrid chimeric receptors, are a useful starting point for identifying the domains involved in ligand binding and signalling. Site-directed mutagenesis may be used to further delineate functional domains through point specific substitutions or deletions (Monteclaro *et al*, 1997)

It is well established that for some GPCRs, such as the β-adrenergic receptor, palmitoylation of cysteine residues in their cytoplasmic tails can modulate their biological activities. Palmitoylation of GPCRs has been shown to affect different

functions of the receptors, such as membrane targeting, signalling properties and recycling. Although it is widely accepted that most (but not all) chemokine receptors display cysteines in their cytoplasmic tails, it is only in that last year that palmitoylation has a role in chemokine receptor function. Blanpain *et* al (2001) have demonstrated that this biochemical modification of cysteine residues is indeed necessary to allow efficient trafficking of CC-chemokine receptor 5 (CCR5). Whether these functions can be generalised to the palmitoylation of other chemokine receptors on other leukocytes remains to be determined.

CC chemokine receptor 2 (CCR2)

Two independent groups simultaneously cloned the MCP-1 receptor, CCR2. CCR2B was identified by Yamagami *et al*, who showed that it encodes a protein of 360 amino acids and shares a high degree of similarity (56%) with CCR1. Cloning by Charo and co-workers culminated in the production of two positive clones corresponding to two splice variants of CCR2: CCR2A and CCR2B. Both isoforms have identical 5' untranslated and transmembrane regions, but have dissimilarities in the alternatively spliced carboy-terminus (Charo *et al*, 1994). This alternative splicing renders CCR2B with 36% homology to the corresponding region of CCR1, however the carboxyl tail of CCR2A bears no similarities to any other known chemokine receptor.

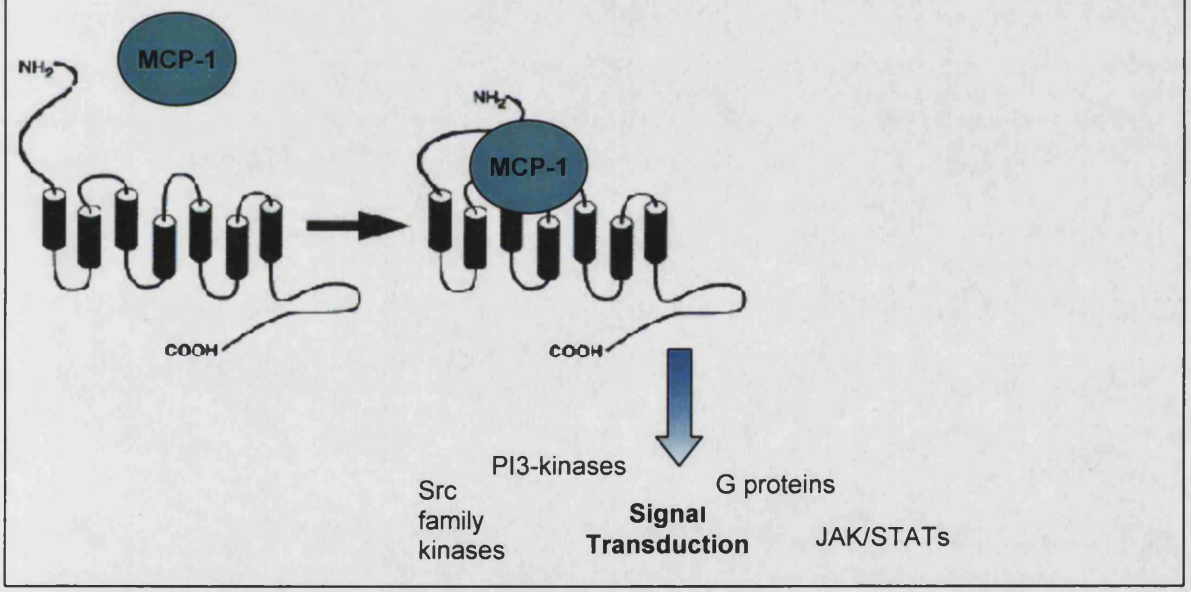
Despite years of intensive research, the process by which CCR2 is activated by its ligands remains a matter of contention. More detailed analyses of the ligand binding domains of CXCR1 and CXCR2 have suggested that multiple regions of the receptor are required to initiate functional interaction. Similarly, several groups have shown that the interaction of MCP-1 with CCR2 involves multiple domains. In particular, the first extracellular loop is thought to contain critical microdomains for ligand binding (Han *et al*, 1999) which is in agreement with a hypothetical model that predicts a two-step mechanism for CCR2 activation (Figure 1.5). In this model, MCP-1 binds first to the amino terminal tail with high affinity before it interacts in the second step (with relatively low affinity) to distal extracellular domains (Monteclaro and Charo, 1996). As a general rule, the N-terminus and extracellular loops of these receptors have been implicated in peptide agonist interactions, and similarly, the C-terminus and the intracellular domains co-operate to bind G proteins and other downstream effectors (Bockaert and Pin, 1999).

A limited heterogeneity is demonstrated in the coupling of CCR2 to G proteins. For example, although CCR2A and CCR2B can couple to the G_i-G$\beta\gamma$-PLCβ_2, these

isoforms exhibit an interesting specificity in their coupling to the α subunits of the G_q class: CCR2B couples to both $G\alpha_{16}$ and $G\alpha_{14}$, whereas CCR2A cannot couple to either (Kuang *et al*, 1996). Similar observations are seen in the coupling of SDF-1 and RANTES to both pertussis toxin sensitive G_i (Hesselgesser *et* al, 1998; Bacon *et* al, 1995) and pertussis toxin-insensitive G_q members of the G protein family (Bleul *et al*, 1996).

As suggested for other chemokine receptors, it has also been proposed that CCR2B undergoes homo- and hetero-dimerisation on ligand binding. A number of different approaches have been used to demonstrate how a complex of at least two receptors are required to induce a functional response. Oligodimerisation in the CCR2 model has been clearly demonstrated, and is thought to induce tyrosine phosphorylation leading to JAK kinase activation, in addition to possible recruitment of Src family kinases, and STAT transcription factors (Mellado *et al*, 1998).

Figure. 1.5. Model of CCR2 Activation



The hypothetical 'two-step, two-site' model of CCR2 activation. MCP-1 receptor activation proposed by Monteclaro and Charo (1997). Initially, the MCP-1 binds with high affinity to 30-35 critical residues at the N-terminal extension of CCR2. *The* 'pseudo-tethered' ligand binds to distal extracellular loops with much less affinity and initiates signal transduction.

Hetero-dimerisation between chemokine receptors has also shown to have functional relevance in agreement with the observations of opioid receptors. There is some biochemical and pharmacological evidence to show that hetero-dimerisation of GPCRs causes synergistic agonist binding and potentiates signalling. With respect to CCR2, hetero-dimerisation not only has a functional significance, but may also provide aetiological significance in diseases associated with this receptor.

A polymorphism reported for the CCR2 receptor, whereby the Val64 is replaced by Ile (CCR2V64I) is associated with a 2-4 year delay in progression to acquired immunodeficiency syndrome (AIDS) (Lee *et al*, 1998). In comparison to other chemokine receptors, relatively few viral strains are known to use CCR2 as a coreceptor to infect cells. Other groups have shown that the mechanism underlying this protective effect may be the ability of the mutant CCR2Val64 to hetero-dimerise with CCR5 and/or CXCR4 (Mellado *et al*, 1999). More recently, this group have shown that hetero-dimerisation of CCR2 not only induces more efficient biological responses in response to MCP-1, but associates with specific signalling pathways, such as recruitment of $G_{q/11}$, a pertussis toxin insensitive G protein.

CCR2: A Key Player in Atherosclerosis

The chronic inflammatory response that typifies atherosclerosis comprises the recruitment of myeloid cells and the transmigration, development of low-density lipoprotein (LDL)-laden macrophages and T cells that contribute to the formation atherosclerotic lesions. For years, MCP-1 has been implicated in monocyte extravasation and plaque formation since high levels are consistently found in macrophage-rich areas of atherosclerotic vasculature (Nelken *et al*, 1991). The most confounding evidence has come from *in vivo* studies using MCP-1-deficient mice which overproduce apolipoprotein (ApoB/MCP-$1^{-/-}$), and are therefore susceptible to atherosclerosis (Gosling *et al*, 1999). These mice were protected from plaque formation even when fed on a high-fat diet, and this is highly suggestive that MCP-1 expression is crucial to the development of atherosclerotic plaques. Furthermore, mice that were defective in both MCP-1 and LDL receptors had over 80% less deposition of lipids in their arteries compared to the control mice. (Gu *et al*, 1998)
These genetic data were further supported by studies using CCR2 knockout mice predisposed to atherosclerosis (ApoE/CCR$2^{-/-}$). Interestingly, the double knockout mice had 50% fewer lesions compared to the controls, indicating that CCR2 is the key receptor involved in the pathogenesis of this disease (Boring *et al*, 1998). However, it should be noted that lesion formation is not completely abrogated in

these models, and so, another recruiting agent is thought to be in operation. (For review of *in vivo* studies into the recruitment properties of MCP-1/CCR2 in atherosclerosis see Peters and Charo, 2001).

CCR11 – An alternative to CCR2?

The orphan receptor PPR1 was originally isolated from bovine papillary tissue although was found to have striking structural similarities to CC chemokine receptor (Matsuoka *et al*, 1993). Further analysis demonstrated that the human homologue of PPR1 did in fact bind members of the MCP family (MCP-1, -2, -3, -4 and eotaxin) with high affinity and also mediated functional responses to MCP-1, -2 and –4. PPR1 has since been designated CCR11 – a functional receptor to the MCP-1 family. (Schweickart *et al*, 2000). The characterisation of this novel receptor opened up a new concept in the MCP research community.

Unlike other chemokine receptors, CCR11 is poorly expressed on lymphoid or myeloid cells, but shows patterns of expression in cells of vascular tissues. Given that MCP-1 and CCR2 are associated with atherosclerosis development, it is reasonable to assume that this may be accompanied by CCR11-mediated events. The result of knockout studies whereby gene mutations are not lethal and result in relatively mild phenotypes may be explained by the complex redundancy of MCP chemokines in the vascular environment.

1.4 Signalling from GPCRs

The phosphoinositide-specific phospholipases (PLCs) play important roles in cellular metabolism including the biosynthesis and degradation of membrane lipids. The catalysis of minor lipid phosphatidylInositol(4,5)P_2 by a PLC is one of the first key events by which more than 100 signalling effectors are known to regulate their target cells. The hydrolysis culminates in two important secondary messengers; Diacylglycerol (DAG) and inositol-1,4,5-trisphopshate ($InsP_3$). Like the other phospholipases, PLC exists in multiple isoforms; PLC-β, PLC-γ and PLC-δ (for review see Rhee and Bae, 1997). Of these isoforms, only PLC-β appears to be sensitive to GPCR-activation.

Stimulation rapidly activates PLC-β_2 and PLC-β_3 isoenzymes, which can occur through the GTP-bound α-subunits of the G_q class of heterotrimeric G proteins or by

the Gβγ subunits (Exton *et al*, 1997; Zhang and Neer *et al*, 2001). The PLC-β enzymes also exhibit differential sensitivities to G protein subunits, and this may contribute to the diversity in the nature and magnitude of the enzyme responses observed. A number of recent studies have provided an idea of domain organisation of $PLC\beta_2$ and have been able to characterise structure, catalysis and cellular localisation of this enzyme (reviewed in Katan, 1998). All of the ten mammalian PLC isoenzymes identified to date are; 1) modular proteins, 2) contain a pleckstrin homology (PH) domain, and 3) a catalytic domain and a C2 domain.

Calcium signalling

The PLC product, $InsP_3$, subsequently binds to receptors on intracellular calcium stores, in particular the endoplasmic reticulum, and induces calcium mobilisation. This pathway has commonly been used to test the responses of ligands (e.g. chemokines, hormones) to their receptors (Baggiolini *et al,* 1997). Increases of $[Ca^{2+}]$ regulate a diverse range of cellular processes ranging from fertilisation to cell death (reviewed in Berridge *et al*, 1999). Calcium ions are utilised as intracellular signalling molecules to control many biological processes and these signals are conveyed by calcium sensors such as calmodulin (CAM) and troponin C (TnC). These proteins are responsible for detecting the Ca^{2+} rise and transducing the information into specific cellular responses.

Protein Kinase C

In hand with $InsP_3$, the other product of $PtdIns(4,5)P_2$ hydrolysis by PLC is diacylglycerol (DAG), which can activate particular isotypes of protein kinase C (PKC). PKC, a major target for tumour promoting phorbol esters, and has been implicated as having a major role in both short term and long term regulation of cellular responses including changes in cell morphology, ion fluxes, differentiation, and proliferation (Exton, 1997). Although PKC is recognised as an important enzyme in many signalling processes, the downstream effects remain poorly defined. The present family of 11 PKC isotypes (α, βI, βII, γ, δ, ε, ζ, θ, ζ, τ/λ and μ) are grouped on the basis their structural and functional considerations (Figure 1.6). Most PKC isotypes display typical DAG (or phorbol ester) dependence for their kinase activity, with only the atypical isotypes (aPKC) being insensitive.

PKC is regulated by two sequential, and equally important mechanisms: (1) phosphorylation triggered by the recently discovered 3-phosphoinositide-dependent

kinase (PDK)-1 and; (2) binding to the lipid second messenger DAG (Le Good *et* al, 1998; Chou *et al*, 1998; Parekh *et al,* 2000). Both of these mechanisms are important in regulating the structure, subcellular localisation, and function of PKC (Dempsey *et al*, 2000). Activation of PKC isoenzymes is stimulated by almost every cell surface receptor and is therefore not a characteristic event exclusive to GPCR-induced signal transduction. However, PKC activation is required for biological functions triggered by GPCRs, such as respiratory burst in neutrophils and cell migration (Li *et al*, 2000) and is involved in receptor phosphorylation and consequent desensitisation (Signoret *et al*, 1997).

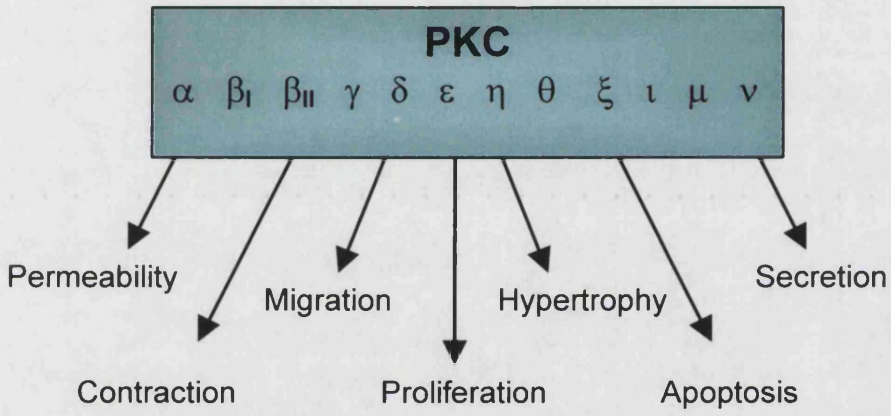


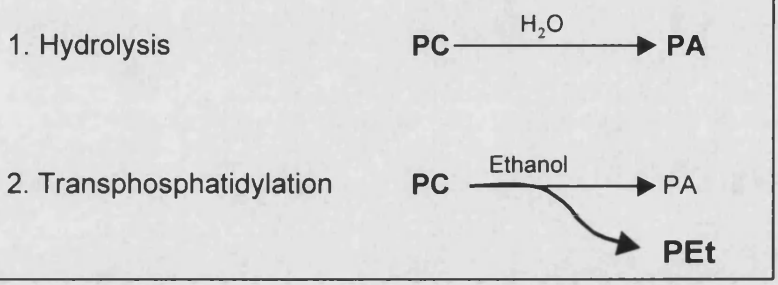
Figure 1.6 .PKC Signal transduction pathway exhibits heterogeneity. Isoenzymes can be divided into subgroups based on structure and cofactor requirements: conventional (α, β_I, β_{II}, γ), novel (δ, ε, η, θ), atypical (ξ, ι) and the recently described (μ, ν).

Phospholipase D

Phospholipase D (PLD) is now recognised as a vital component of signal transduction pathways, vesicular trafficking (Jones *et al,* 1999; Exton, 1999), and cell transformation (Ohguchi *et al*, 1999). PLD is a ubiquitous enzyme that hydrolyses membrane phospholipids especially phosphatidylcholine (PC) to generate phosphatidic acid (PA) and choline. Choline is not thought to serve as a second messenger, since it remains in high concentrations in resting cells (McPhail, 1999). PA, however, has been implicated as a biologically active molecule and can be further metabolised by PA-phosphohydrolase to form DAG, a protein kinase C activator. PLD attacks the phospholipid substrate to generate a transient phosphatidyl-PLD intermediate. With water as an acceptor, PA is generated; however, in the presence of a primary alcohol PLD preferentially catalyses a unique

transphosphatidylation reaction generating phosphatidyl-alcohol (Figure 1.7). The production of the phosphatidyl-alcohol has been extremely useful for the detection of PLD activation since unlike PA (which can also be produced by DAG kinase and by acylation of glycerol-3-phosphate) a phosphatidyl-alcohol is uniquely formed by PLD. Unlike PA that can be further metabolised to DAG and lyso-PA, a phosphatidyl-alcohol remains metabolically stable. (Liscovitch *et al,* 1999).

Figure 1.7 Product of PLD catalysis depends on acceptor molecules



A growing body of evidence is now emerging that is beginning to establish potential links between phosphatidylinositol 3-kinase (PI3K) and PLD. This has risen form the observation that at least three Ca^{2+}-dependent PLD isoenzymes may exist with specificity for PtdIns(3,4,5)P_3, the major lipid product of PI3K (Ching *et al* 1999). Also, activation of small GTP-binding protein, Ras, via PI3K is thought to lead to the activation of the Ras effector molecule Ral, which itself has been implicated in PLD activation (Jiang *et al*, 1995). The most interesting link is that emerging between PI3K and members of the ARF family of proteins (Frohman *et al*, 1996; De Camilli *et al*, 1996). ARFs (consisting of ARF1-6) cycle between inactive GDP- and active GTP- states which are regulated by ARF-GEFs (ARF-guanine nucleotide exchange factors), and ARF GTPase activating proteins (ARF-GAPs).

It is the family of ARF-GEFs (including GRP-1, ARNO and cytohesin-1) and their interactions with PI3K that has generated such interest. ARF-GEFs contain a PH domain that binds to PtdIns (3,4,5)P_3 in preference to other phosphoinositide lipids (Ching *et al*, 1999), and facilitate its own translocation to the cell membrane. The use advent of green fluorescent protein (GFP) imaging of ARF-GEF constructs has helped to characterise the PI3K-dependent regulation of ARFs and subsequent PLD activation (Venkateswarlu *et al*, 1998, Hmama *et al*, 1999).

1.5 Phosphatidylinositol 3-kinase- Dependent Signalling Cascades

The PI3-kinase family

Membrane lipids do not only have a structural role in the mammalian cell, but are also implicated in receptor stimulated signalling. The 3-phosphorylated inositol lipids assume roles as second messengers by interacting with a multitude of cellular proteins. The generation of these lipids and their subsequent impact of signalling pathways influence many fundamental cellular activities. The term 'PI3-kinase' is now applied to a rapidly growing family of proteins that catalyse the conversion of PtdIns, PtdIns(4)P and PtdIns(4,5)P_2 to 3-phosphorylated lipids, PtdIns(3)P, PtdIns(3,4)P_2 and PtdIns(3,4,5)P_3, respectively, by phosphorylating the D-3 position of the inositol ring (Figure 1.8). PtdIns(3)P is constitutively present in eukaryotic cells, and its levels are unaltered upon stimulation. PtdIns(3,4)P_2 and PtdIns(3,4,5)P_3, on the other hand, are almost absent from resting cells, but cellular stimulation with a variety of ligands results in a marked rise in intracellular concentration. This is highly indicative of a likely second messenger function (reviewed in Stephens *et al*, 1993).

The multiple isoforms of PI3Ks can be divided into three classes on the basis of their *in vitro* substrate specificity, structure, and likely mode of regulation. All PI3K catalytic subunits share a homologous region that consists of a catalytic core domain (HR1; homologous region 1) linked to HR2 (know as the PI kinase homology domain) and a C2 domain.

The Class I PI3-Ks

Class I PI3Ks are heterodimers consisting of a catalytic subunit and a regulatory subunit. *In vitro* these PI3Ks phosphorylate and utilise PtdIns, PtdIns(4)P, and PtdIns(4,5)P_2 as substrates. However, in intact cells, PtdIns(4,5)P_2 appears to be the substrate of choice. This class of enzymes can further be divided into class 1_A and class 1_B, which signal downstream of tyrosine kinases and G protein-coupled receptors, respectively. All mammalian PI3Ks from this class interact with active GTP-bound Ras, although the role of this interaction in PI3K signalling is not understood. Numerous studies have shown that all members of this class form heterodimeric complexes with adaptor proteins that link them to upstream signalling events (Vanhaesebroeck, 1997).

Figure 1.8 Structure of Phosphatidylinositol and the cycle of phospholipid generation by the action of PI 3-kinases

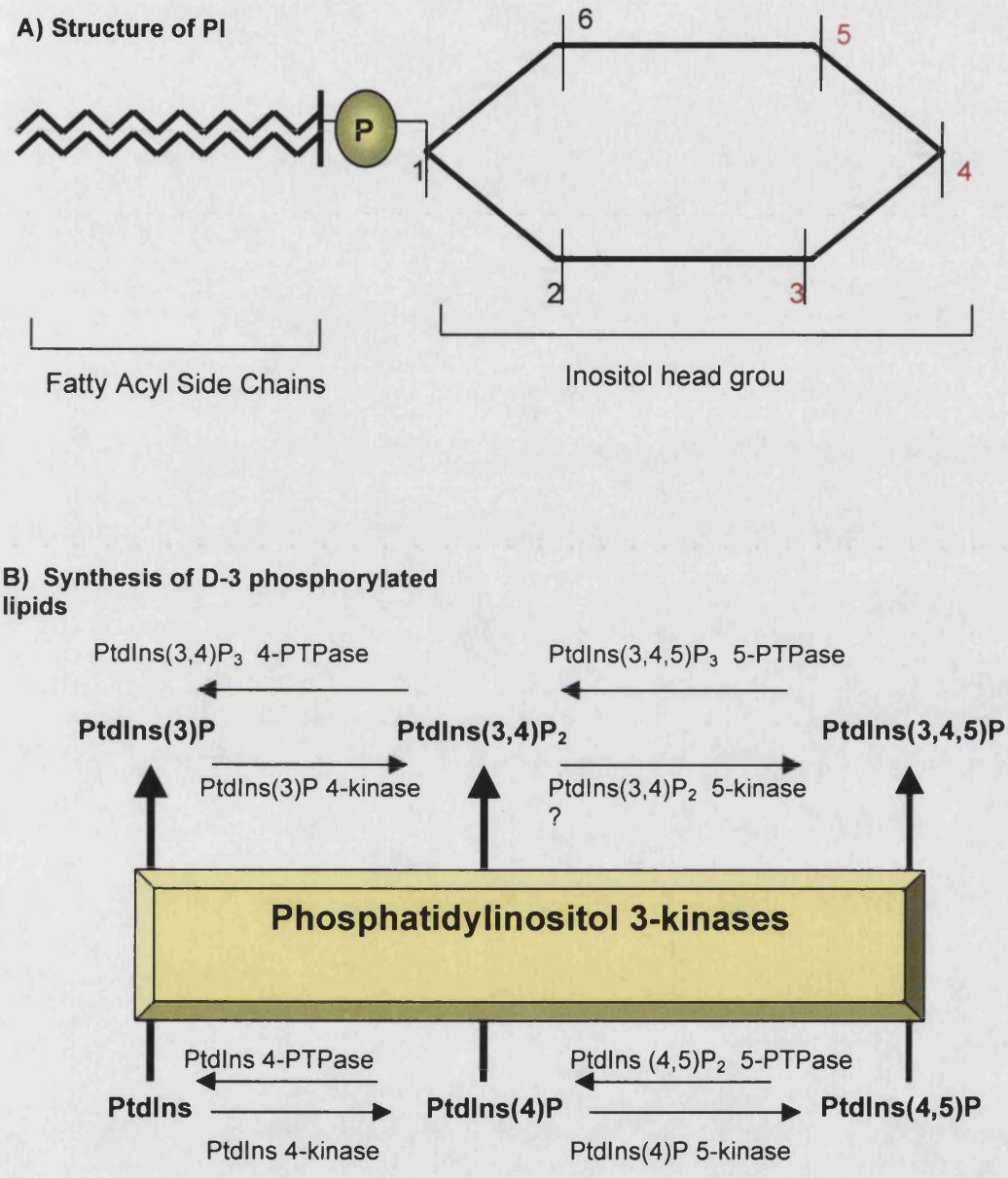


Figure 1.8. **A)** The positions 3, 4, 5 indicated in red signify those which can receive a phosphate group, however, only position 3 phosphorylation occurs through PI3Ks. The enzyme activity of PI3K phosphorylates the 3'-hydroxyl group on the inositol ring of PtdIns (or indeed that of PtdIns(4)P and PtdIns(4,5)P_2) by the transfer of the terminal phosphate group from ATP. **B)** The tightly regulated cycling of D-3 phosphoinositide lipids under the control of PI 3-kinases, related kinases, and lipid phosphatases.

Class 1_A PI3Ks

Class 1_A PI3Ks are 100-130 kDa proteins that interact with adaptor subunits containing Src homology-2 (SH2) domains. Activation of tyrosine kinases in receptors and receptor-associated adaptors results in the generation of phosphorylated tyrosine residues. It is to these residues, preferably in a Y(P)xxM motif (where x is any amino acid), that the afore mentioned SH2 domains on the adaptor subunits are likely to bind. This phosphotyrosine binding is thought to facilitate the translocation of the PI3K from the cytosol to the membranes, ensuring proximity to their lipid substrates and small molecular weight G-proteins.

Under resting conditions, p85 serves to stabilise p110 protein and inhibit kinase activity. This inhibitory effect is alleviated by binding of SH2 domains of p85 to tyrosine phosphorylated peptide, receptors or linker molecules (Cuevas *et al.* 2001). Mechanisms for the recruitment of PI3K not involving tyrosine phosphorylation events have also been reported (Prasad *et al*, 1993). Most p85 gene products also include a Src-homology-3 (SH3) domain, two proline rich domains and a break point cluster (BCR)-homology domain (BH) all of which are thought to be implicated protein-protein interactions (Wymann and Pirola, 1998).

To date, eight different adaptor subunits for class 1_A catalytic subunits have been described (seven in mammals and one in *Drosophila*) which are generated by expression and alternative splicing of three separate genes, namely p85α, p85β and p55γ. All of which make functional complexes with the p110 catalytic subunit. Mammals have three class 1_A p110 isoforms, p110α, β and δ, all encoded by different genes. p110α and β are ubiquitously expressed in mammalian tissue, in contrast to p110δ that is mainly found in leukocytes (Vanhaesebroeck *et al.* 1997). So far, there is no evidence of preferential coupling between any of PI3K adaptor molecules and p110 catalytic subunits, however, it is reasonable to assume that differences in function or regulation may exist due to tissue specificity.

At least one of these class1_A PI3K isoforms is expressed in all of the mammalian cell types studied, and engagement of every receptor known to induce tyrosine kinase activity is shown to activate class 1_A PI3K This activity can be induced by receptors with intrinsic tyrosine kinase activity or non-receptor tyrosine kinases, such as Src-family kinases (reviewed in Wymann and Pirola, 1998).

The Class 1_B PI3Ks

Several years ago, it was shown that haematopoietic cells possess a PI3K that can be directly stimulated by Gβγ heterodimers. This distinct lipid kinase, now referred to as PI3-kinase-γ, is the only characterised member of the class 1_B G protein-activated PI3K, and consists of a unique 101kDa regulatory subunit and a distinct 110kDa called p110γ. Unlike the class 1_A PI3Ks, the p110γ subunit does not have a site that binds to the regulatory p85, but instead a Ras-GAP homology region, which may fold to form a PH domain, that contributes to form a common Gβγ effector region (Stoyanov *et al*, 1995). Direct activation of the p101/p110γ PI3K is then mediated by the Gβγ subunit. Although several reports show that the activation of p110γ are substantially amplified by the presence of p101, some groups suggest that p110γ alone can be catalytically activated by Gβγ and have clear biological effects (Stephens *et al*, 1994). Therefore, the function of the p101 non-catalytic subunit of PI3K-γ has yet to be identified.

The physiological roles of PI3Kγ have been clearly defined particularly in the context of linking GPCR stimulation to the formation of PtdIns(3,4,5)P_3 and the subsequent activation of other downstream effectors such as PKB, ribosomal protein S6 kinase, and MAPKs. Several studies based on the generation of p110γ null mice have shown this enzymes' impact on cellular signalling to be crucial for several biological inflammatory responses such as thymocyte development, T cell activation, neutrophil migration and oxidative burst (Okada *et al*, 1994, Sasakdi *et al*, 2000).

The dynamic nature of class I PI3K signalling seem to be achieved by a second enzymatic function, *i.e.*, protein-serine kinase activity. Bondeva *et al*, 1998 reported that cellular signalling bifurcates at the level of PI3Kγ. Whereas phosphatidylinositides target PKB through the lipid kinase activity of PI3K-γ, the protein kinase ability of this enzyme concurrently phosphorylates the extracellular signal regulated kinase/mitogen-activated protein kinase pathway (ERK/MAPK). In this respect, reports have suggested that the p101 subunit was critical for supporting p110γ-induced PKB and c-Jun amino terminal kinase activation (JNK), but had little consequence on the ERK/MAPK activation. This is highly suggestive that p101 may differentially modulate the lipid and protein kinase activity of p110γ.

Class II PI3Ks

Class II PI3K include PI3K-C2α (mcpk, p170), PI3K-C2β (HsC2-PI3K) and PI3K-C2γ, and are distinguished from other PI3K isoenzymes by the presence of two tandem domains at their carboxyl terminus: the Phox (PX) and the C2 domains. The PI3K-C2α PX domain itself has been shown to associate selectively with liposomes containing PtdIns(4,5)P_2 (Xu *et al.*, 2001). The second important feature is the C2 domain - a phospholipid-binding molecule that can confer a Ca^{2+} sensitivity. The role of this motif is also not understood, although the diversity in biochemical function mediated by C2 domains has been clearly demonstrated. C2 domains of synaptotagmin, protein kinase C and phospholipase C can bind a variety of ligands, including Ca^{2+}, phospholipids, inositol polyphosphates and intracellular proteins (Ponting and Parker, 1996).

The nature of receptor-linked activation pathways to these isoenzymes is only recently becoming elucidated. PI3K-C2α plays a signalling role downstream of both the receptor for the CC chemokine MCP-1 (Turner *et al*, 1998) and the insulin receptor (Brown *et al*, 1999), and engagement of epidermal growth factor and platelet-derived growth factor receptors result in activation of both PI3K-C2α and PI3K-C2β (Arcaro *et al*, 2001). The observation that PI3K-C2α is concentrated in the trans-golgi-network and is present in clathrin-coated vesicles also suggests that this PI3K may play a role in regulating adaptor protein function (Domin *et al*, 2000)

Studies of the murine (Virbasius *et al,* 1996) and human enzymes (Domin *et al* , 1997) show their *in vitro* substrate specificity is restricted to PI and PI(4)P, and that they cannot utilise PI(4,5)P_2. However, it should be noted that PI3K-C2α might have a different substrate specificity in intact cells to that observed under *in vitro* conditions. Interesting, of all the mammalian enzymes, PI3K-C2α remains the most refractory to PI3K inhibitor, wortmannin (Domin and Waterfield, 1997).

Class III PI3Ks

The classes III PI3Ks are homologous to the Vps34p, the only PI3K identified in yeast. These enzymes are restricted in their substrate specificity in that they only phosphorylate PtdIns, and are probably responsible for the majority of Ptd(3)P in cells. Since the cellular levels remain constant, and are not altered upon cellular stimulation. It is assumed that the involvement of class III PI3K in intracellular trafficking is not a product of cellular stimulation (Reviewed in Odorizzi *et al*, 2000).

1.5.2 The Phosphoinositide Lipids Binding Molecules

It is generally assumed PI3K exerts its biological effects by promoting the localisation or activity of other downstream molecules. This has been further supported by the characterisation of protein motifs that are known to specifically bind lipids. To focus the discussion of 3-PI targets, we now consider three structurally distinct PI-binding domains (FYVE, PX, and Pleckstrin homology) for which the structure and nature of interaction has been defined at the molecular level.

Pleckstrin Homology Domains

Pleckstrin homology (PH) domains are globular protein domains of around 100 amino acids that can bind phospholipids. PH domains and the structurally related phosphotyrosyl binding (PTB) domains are present in many signalling proteins, such as kinases, phospholipases, and adaptor proteins. The core PH domain structure is made up of two β-sheets (of four and three anti-parallel β-strands), that is capped on one side by a C-terminal α-helix. The inositol head group of the ligand becomes sandwiched between the loops at the end of the barrel distal from the C-terminal α-helix (Lemmon and Ferguson, 2000). The relevance of phosphoinositide binding to these domains is unclear, however, several of these motifs have now been shown to bind to PtdIns(4,5)P_2, Ins(1,4,5)P_3 and products of PI3-K, such as PtdIns(3,4)P_2 and PtdIns(3,4,5)P_3 (Rameh *et al*, 1995, Saito *et al*, 2001). The PH domains of the serine threonine kinase PKB and phosphoinositide-dependent kinase-1 (PDK1), guanine nucleotide exchange factors (GEFs), Bruton's tyrosine kinase (Btk) family kinases and phospholipase C provide examples of this.

A sequence motif for PI3-K product binding by PH domains

Consensus	**ϕxKx**	**Gx**	***K***	**x**		**xRxRxF**		
		A	**R**			**K**	**L**	
		S						
		P						

In the above consensus sequence 'ϕ' represents an amino acid with a hydrophobic side chain, 'x' represents any amino acid; and '*'represents any number of amino acids (0 or more).

Table 1.4 Predicted 3' Phosphoinositide binding preferences. PH domain-containing proteins

PtdIns(3,4,5)P_3	PtdIns(3,4)P_2/ PtdIns(3,4,5)P_3	PtdIns(3,4)P_2
Grp-1	DAPP	TAPP1
Btk	PKB	TAPP2
Centaurin-α	Sbf1	
DOS		
Gab1		
$Gap1^{IP4BP}$		
Gap1m		
PDK-1		
↓ ↓		
Transient recruitment to the membrane	**Longer-lived membrane association**	

The FYVE Domain

In 1998, several groups reported the identification of a PI(3)P-binding molecule - the FYVE domain - that is found in various proteins including some that are implicated in endosomal function. The FYVE domain of human early endosome autoantigen 1 (EEA1) was first identified as a motif responsible for binding exclusively to Ptd(3)InsP (Patki *et al*, 1998), and now more than 30 mammalian protein containing this motif have been identified. Instead of a targeting role, it seems that the FYVE domains may execute a regulatory role on the functional activities of several proteins, possibly through conformational changes upon their interaction with PtdIns(3)P (Xu *et al*, 2001).

The Phox Domain

The Phox Homology (PX) domain, a 125 residue module, has been observed in numerous human and yeast proteins including kinesins, phospholipases PI3Ks and SNAREs (Cheever *et al.*, 2001). The recent establishment that the PX domain interacts with PtdIns(3)P and other phosphoinositides suggests another mechanism by which phosphoinositides regulate cellular events. Most proteins that contain the PX domain are involved in membrane trafficking (e.g. PLD) cytoskeletal organisation and protein sorting (e.g. SNXs), and signal transduction (e.g. PI3K-C2α, β, γ) but the

function of this domain remains unclear. The conservation of key structural and binding site residues across the diverse PX family does, however, indicate a shared fold and phosphatidylinositol recognition function.

1.5.3 Downstream Effectors of PI3K.

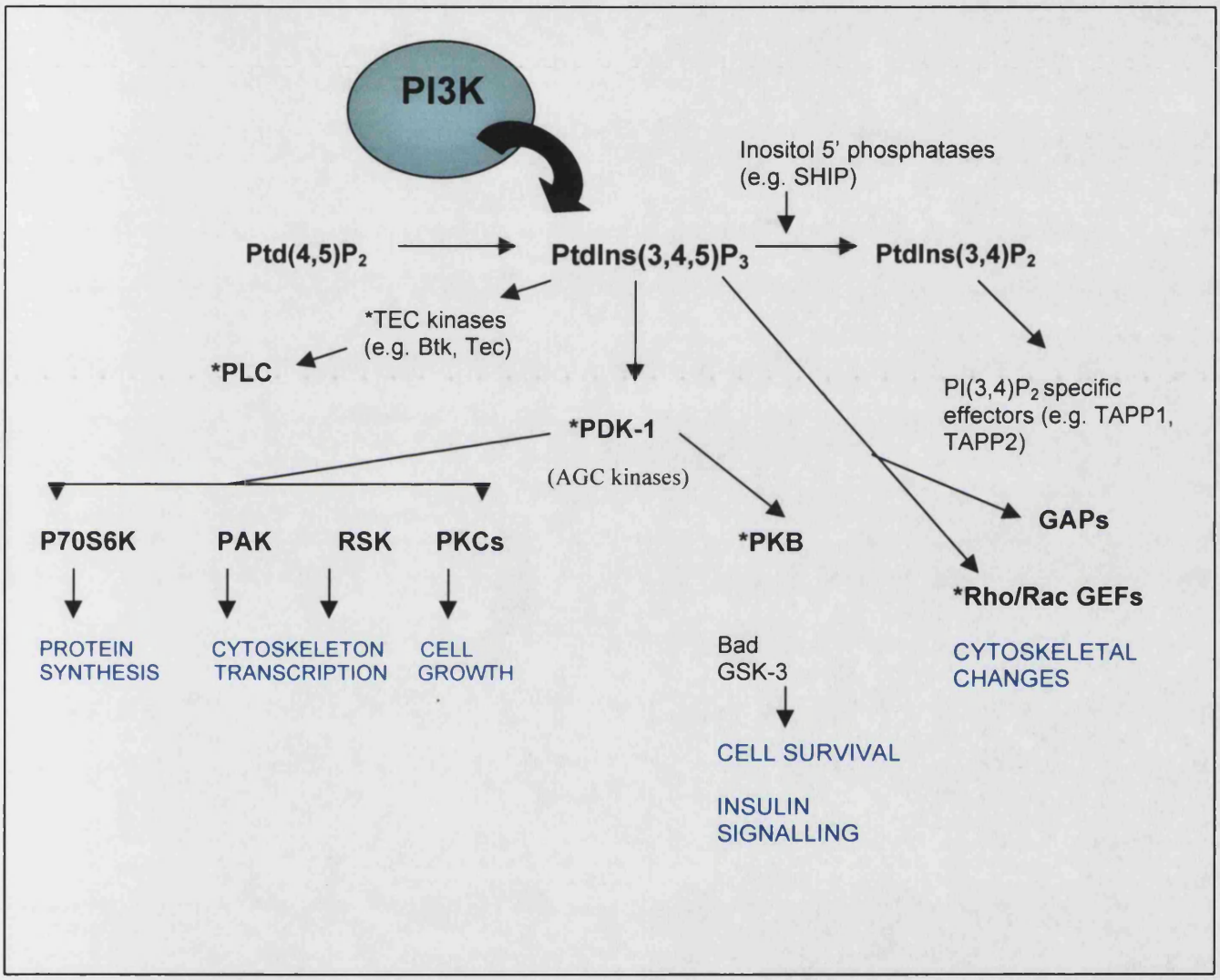


Figure 1.10. The cellular effects of PI3-Kinase. Summary of the best-characterised pathways initiated by the major lipid products of PI 3-kinase. Asterisked proteins have a PH domain that directly binds to PtdIns(3,4,5)P_3.

Protein kinase B

How D-3 lipids transduce the effects of agonists of PI3K was unclear until the recent discovery that several protein kinases become activated upon exposure to these molecules. Some 15 years after the identification of the AKT8 retrovirus the cellular homologue of v-Akt was cloned by several groups and found to be a 57kD a protein serine threonine kinase, currently referred to as Akt or protein kinase B (PKB) (Coffer and Woodgett, 1991). Analysis of the PKB sequence reveals a multi-domain protein, and includes an N-terminal PH domain assumed to be responsible for binding PI-3P lipids (Haslam *et al*, 1993).

Although there is still debate about the precise role and nature of PI-3P lipids in the activation of PKB, it is clear that most growth factor/cytokine-signalling pathways require PI-3P lipid formation for PKB activation. Activation of PKB is accompanied by phosphorylation, and this appears to be the required step in its induction. This may occur by transphosphorylation by other kinases or by autophosphorylation following activation. For PKBα, the issue has been resolved by the identification of two crucial phosphorylation sites; Thr^{308} and Ser^{473}, and this suggests that there is dual regulation. Sufficient levels of PI-3P lipids promote the recruitment of all the required machinery to the plasma membrane, thus, placing the PKB multimer in the correct proximity for interactions with 'third party' kinases. The enzymes specifically identified for the phosphorylation Thr^{308} and Ser^{473} were tentatively named PtdIns(3,4,5)P_3-dependent kinase-1 and -2 (PDK-1 and 2), respectively, and were thought likely to be regulated in an analogous manner since phosphorylation of the two sites on PKB is tightly co-ordinated (for review see Coffer *et al*, 1998; Lawlor and Alessi, 2001).

Recent work indicates that PDK2 may in fact be a 'modified' PDK1 since the kinase domain of PDK-1 was found to bind to carboxy-terminal residues of PKC-related kinase-2 (PRK2), termed the 'PDK-1 interacting fragment' (PIF). The interaction has been shown to occur through a 24-amino acid fragment of PIF that encompasses the hydrophobic motif of PRK2 and binds to a pocket on the kinase domain of PDK1 -the 'PIF-binding pocket' (Biondi *et al*, 2001). In the presence of PIF, PDK-1 and PtdIns(3,4,5)P_3, PKB was shown to be phosphorylated at Thr^{308} and Ser^{473} (Balendran *et al*, 1999). In the light of this research, it is unclear whether the activity of PKB comprises PDK1 complexed to a 'PIF-like' protein and/or PDK1 complexed to a distinct PDK2. It has since been suggested that following phosphorylation of PKB at Thr^{308}, PKB autophosphorylates itself at Ser^{473}. However, the finding that Ser^{473} is

phosphorylated in PDK1-deficient ES cells in the absence of Thr^{308} argues against this possibility (Williams *et al*, 2000).

Amino acid sequences very similar to those surrounding Thr308 and Ser473 in PKB are conserved in all members of the 'AGC' Ser/Thr kinases. This group of enzymes includes PKA, PKG, PKC, and PKB isoforms, in addition to P70S6K, p90-ribosomal kinase, and the serum- and glucocorticoid-induced protein kinase (SGK). These enzymes mediate many of the cellular effects of extracellular agonists by phosphorylating key regulatory proteins, and like PKB, are modulated by PDK-1 phosphorylation (Vanhaesebroeck and Waterfield, 1999).

Activated PKB promotes cell survival by phosphorylating and inactivating several apoptosis-mediating proteins such as BAD, caspase-9 and forkhead transcription factor (Brunet *et al*, 1999). Until recently, the molecular mechanisms of induction of apoptosis have been ill understood. However, the use of PI3K inhibitors has implicated this pathway in the balance between cell survival and apoptosis (Yoa and Cooper, 1995; Burgering and Coffer, 1995). Such studies have led to the investigation of the role of PKB in the regulation of these processes. Several groups also have provided the link between PKB and intracellular glucose transport, glycolysis, protein synthesis, and perhaps most interestingly, the synthesis of glycogen via GSK-3 (Sheppard *et al*, 1996).

Glycogen synthase kinase-3

The principle substrate of PKB is glycogen synthase kin*ase* α/β (GSK-3) which was initially identified as the enzyme that regulates glycogen synthesis in response to insulin (Cross *et al*, 1995). Initially GSK-3 was shown to phosphorylate sites in glycogen synthase, which are specifically dephosphorylated when insulin induces synthase activation that ultimately leads to glycogen synthesis. A more general role for GSK-3 is suggested in its growing number of substrates, including several transcription factors and translation initiation factors. In addition, GSK-3 has been implicated in the regulation of cell fate in *Dictyostelium* (Harwood *et al*, 1995) and is a component of the Wnt signalling pathway required for *Drosophila* (Sigfried *et al,* 1992) and *Xenopus* development (Dominguez *et* al, 1995). Thus, GSK-3 appears to play multiple roles in cell regulation.

Several pathways have been proposed to regulate GSK-3 phosphorylation (including growth factor-induced PKC activation, $p90^{Rsk}$ activation, and p70S6-kinase), however, the pathway regulated by PI3K-dependent PKB remains the best characterised (Weeren *et al,* 1998). Although GSK-3 can be directly phosphorylated and inactivated by PKB, it must be remembered that GSK-3 can also be activated independently of this PKB, (for instance, through the Wnt pathway) (Miller and Moon, 1996).

1.5.4 Approaches to Assess the Functional Significance of PI3-kinase

Pharmacological Approaches

The availability of inhibitors has provided a useful pharmacological approach with which to assess the functional relevance of PI3K activation to cellular signalling. The fungal metabolite Wortmannin first received widespread attention as an inhibitor of the agonist-induced respiratory burst in neutrophils (Baggiolini *et al*, 1987). It was subsequently found to inhibit other pathways including PLC and PLD (Bonser *et* al, 1993), and pleckstrin phosphorylation in platelets (Yatomi *et* al, 1992). However, at nanomolar concentrations (<100nM) wortmannin is a potent inhibitor of PI3K and has been used extensively to illustrate the role of this enzyme in signal transduction processes. Several groups have since identified the critical residues required for the action of this inhibitor and have shown that wortmannin covalently modifies the Lys-802 on the p110α subunit of PI3K, the key residue involved in the phosphate transfer reaction (Wymann *et al.* 1996).

It is clear, however, that various isoforms of PI3K exhibit different sensitivities to wortmannin. The p85/p110 heterodimer is very sensitive, demonstrating an IC_{50} of just 10nM, as is the PtdIns-specific PI3K kinase. In contrast, much higher concentrations (IC_{50} - 46nM) are needed to inhibit the activation of PI3K-γ (Wolscholski *et al,* 1994. Stephen *et al*, 1994). Other PI3K isoforms such as PI3K-C2α are appear relatively insensitive to treatment with wortmannin (Turner *et al*, 1998).

Recent studies have highlighted the limitations of interpreting results using such inhibitors. These relate to the demonstration that wortmannin (at concentrations normally used to inhibit PI3K) has the ability to block the action of at least two other enzymes, namely PtdIns 4-kinase and PLA_2 (Cross *et al*, 1995). Such effects may have profound disruption on several other pathways, and have possible effects on

biochemical and functional assays. Nevertheless, many of the results obtained using wortmannin have been reproduced using the structurally unrelated PI3K inhibitor, LY290042, and strongly suggest that that the actions of wortmannin are specifically due to PI3K inhibition.

Molecular Approaches

With the advances in modern molecular technology, more elegant alternatives to pharmacological agents been developed. Dhand *et al*, were one of the first groups to construct a dominant negative construct of a PI3K isoform. A p85 deletion mutant (Δp85) lacking the inter-SH2 region prevents the interaction between catalytic and regulatory subunits, and renders the enzyme inactive. When expressed in excess of endogenous PI3K, this construct behaves as a dominant negative (Hara *et al,* 1994; Dhand *et* al, 1994). Numerous groups have demonstrated this as a useful tool for observing the role of PI3K in receptor-mediated responses, such as IL-3-stimulated signal transduction in BaF cells (Craddock *et al,* 1999, 2001) and EGF-stimulated DNA synthesis in hepatocytes (Kong *et al.* 2000).

Constitutively active PI3Ks have also been developed. In these constructs, the p110 catalytic subunit is a chimera that is covalently linked to the p85 subunit via the inter-SH2 domain. The constitutively active mutant has been shown to activate a number of pathways such as the p70S6-kinase and Ras/MAPK (Weng *et* al, 1995) and has clearly demonstrated the interaction of PI3K with GLUT4 in glucose transport (Fevert *et al*, 1998).

Genetic Modification

The biological roles of PI3K isoforms are beginning to be elucidated by gene targeting: mice lacking p110γ are viable and fertile but have diminished neutrophil and lymphocyte function (Sasaki *et al*, 2000; Hirsh *et al*, 2000). Conversely, mice lacking p85α are not viable, and studies using $p85\alpha^{-/-}$ stem cells have shown that $p85^{-/-}$ B cells are functionally deficient whereas $p85^{-/-}$ T lymphocytes are completely normal (Fruman *et al*, 1999). Mast cells lacking p85α have a defect in stem cell factor (SCF), but exhibit normal signalling by the high-affinity receptor for IgE (Li-Kuo *et al*, 2000).

Table 1.5 Inhibition of PI3K in functional settings

Method	Example	Advantage	Disadvantage
Gene deletion in animal models	$PTEN^{-/+}$ mice $PI3K\gamma^{-/-}$ mice P85α	Physiological system Allows whole organ effects to be studied	None human Gene disruption may not abrogate all gene products May not target all isoforms encoded by distinct genes
Pharmacological inhibitors	Wortmannin LY-290042	Ease of use in model systems. Specific to PI3K.	Not specific to isoforms. Possible toxicity. Non-specific effects.
Micro-injections of neutralising antibodies	p110 isoform neutralising antibodies	Allows specific isoforms function as to be dissected	Effects from injection. Limited to small number of cells
Molecular inhbition using transfection.	Dominant-negative mutants (e.g. Δp85)	Allows isoforms-specific functions to be assessed. Can be manipulated so effects may be turned on or off.	Effects from transfection procedure

1.5.5 Lipid Phosphatases - Negative Regulators of PI 3-Kinases

Considering the impact of PI3K on many cellular functions, it stands to reason that there should be an effective mechanism to restrain the levels of lipid products and maintain the biochemical milieu of the cell. Tight control of the levels of PI 3-K products, namely PtdIns(3)P, PtdIns(3,4)P_2, and PtdIns(3,4,5)P_3, is required for signal regulation, and is achieved, in most part, by two important phosphatases (Figure 1.11). For the sake of simplicity, let us just consider the fate of PtdIns(3,4,5)P_3, and its PtdIns(3,4,5)P_3-driven effector pathways: There are two characterised routes for its degradation. The first involves the degradation to PtdIns(4,5)P_2 by 3-phosphatase, PTEN. The second achieved through SH2-domain-containing inositol polyphosphate 5-phosphatase (SHIP), that catalyses the conversion of PtdIns(3,4,5)P_3 to PtdIns (3,4)P_2 (for reviews; Cantley and Neel, 1999; Rohrschneider *et al,* 2000).

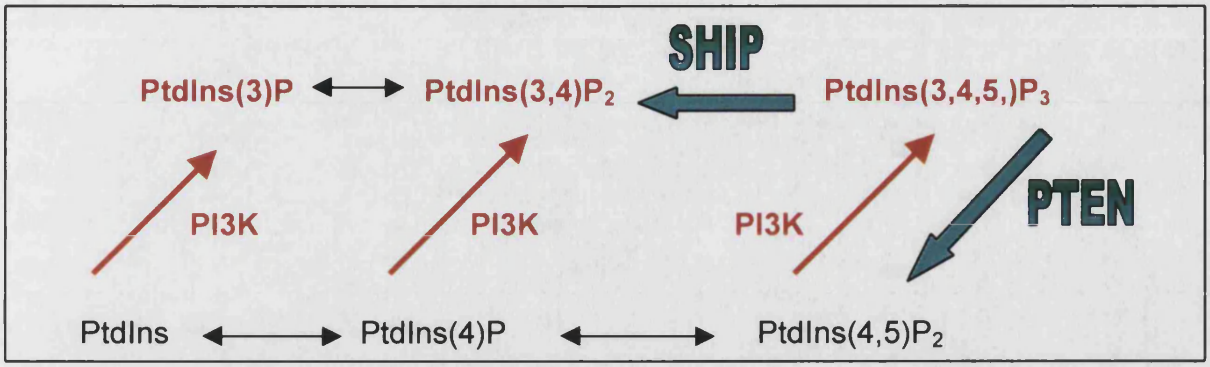


Figure 1.11 Regulation of PI3K products by lipid phosphatases. Selective 3'- and 5'-phosphatases, PTEN and SHIP, regulate the state of phosphorylation of the phosphoinositide lipids. PtdIns, PtdIns(4)P and PtdIns(4,5)P_2 can potentially serve as substrates for different PI3Ks. SHIP and PTEN rapidly degrade the 3' -phosphorylated lipid products of PI3K. This negative regulation helps to maintain equilibrium within the intracellular lipid pool.

PTEN (phosphatase and tensin homologue deleted on chromosome ten) acts as a tumour suppressor and its gene is mutated in a multitude of human cancers. Several papers, including those by Susuki *et al* (1998), now establish a link between PI3K/AKT pathway and cancers via defects in PTEN (Di Christofan *et al*, 1998). Cells lacking PTEN have elevated levels of PtdIns(3,4)P_2 and PtdIns(3,4,5)P_3 and constitutively active PI3K. It is becoming increasingly clear from PTEN knockout models that defects in 3-phosphatase-driven regulation of the PI3K has dramatic effects on the apoptotic and growth regulation, integrin signalling, and cytoskeletal organisation (Tamura *et al*, 1999).

Similarly, an effective negative feedback mechanism is achieved through the activation of SHIP. The regulatory effects of SHIP are mediated by the dephosphorylation of inositol lipids on the D-3 position, and in this case PtdIns(3,4,5)P_3 would be converted back to PtdIns(4,5)P_2. As with PTEN, loss of SHIP function results in an impaired and unbalanced immune response. Mast cells from SHIP$^{-/-}$ animals exhibit greater PKB phosphorylation (as measured by Ser/Thr phosphorylation, and GSK-3 phosphorylation), and levels remain high for extended times following stimulation (Liu *et al*, 1999). These knockouts also show a resistance to apoptotic signals, which is consistent with the function of PKB in this process.

1.6 The Mitogen-Activated Kinase Pathway

The Ras/Raf/ERK pathway

Much progress has been made with respect to the identification of signal transduction pathways involved in gene expression. Of great significance are the so-called mitogen-activated protein kinase (MAPK) proteins. Activation of the different MAPK signalling pathways ultimately results in the direct or indirect phosphorylation and activation of various transcription factors and alterations in gene expression. The MAP kinases represent a point of convergence for cell surface signals regulating cell growth and division. These enzymes comprise a family of serine/threonine kinases, which include (1) The extracellular signal-regulated kinases (ERKs): ERK1 and ERK2; and, (2) The stress-activated protein kinases; the Jun N-terminal kinase (JNK) and p38. MAP kinases are apparently regulated by highly conserved upstream protein kinases that receive signals from effectors acting at the plasma membrane. Extensive investigation demonstrates that in the mammalian ERK1/2 pathway, through interaction with Raf proteins, Ras can activate the dual functioning threonine/tyrosine kinases, MAP/ERK kinases (MEK 1/2), which go on to phosphorylate ERK-1/2 (Downward, 1998). Whereas both Raf-independent and Ras-independent signalling has been reported (Gangarosa *et al*, 1997, Takeda *et al*, 1999), the Ras/Raf/MEK/ERK pathway provides a common route by which growth factor receptor signals converge activate transcription factors such as AP1.

Stress-Activated Protein Kinases

The 'stress-activated' MAPKs include the Big MAPK (BMK/ERK5), the p38 MAPKs and the JNK MAPKs which also phosphorylate transcription factors. The JNKs were first identified on the basis of its capacity to bind to the c-Jun trans-activation domain (Hibi *et al*, 1993). There are two well-characterised proteins, JNK1 (46kDa) and JNK2 (55kDa) which are distant relatives of the MAP kinases. Many substrates are phosphorylated by the JNKs including c-Jun, ATF2, Elk-1 and NFAT that help to regulate gene expression in response to cytokines, growth factors, and other cellular stresses. The activity of JNKs is stimulated by UV radiation, hyperosmolarity and inflammatory cytokines (Review: Harper and LoGrasso, 2001), which is suggestive that the JNK pathway is activated in cellular response to stress. It is interesting how such diverse stress signals appear to activate a common pathway, and given the diverse roles of JNKs (e.g. modulators of T cell activation or neuronal apoptosis), the search for a critical target in this pathway has generated much attention.

P38 is most strongly activated by proinflammatory cytokines and environmental stresses. It is regulated by the upstream kinases MKK3, MKK6 and MKK4, and has been shown to activate a number of downstream substrates including transcription factors ATF2 and PHAS-I and kinases such as Mnk1 and the recently cloned p38-related/activated protein kinase (PRAK) (New *et al*, 1998). A seemingly endless number of extracellular stimuli have been shown to activate the p38 pathway in many systems, and its relative role in cellular function has been made clearer with the use of Glaxo-SmithKline inhibitors, SB203580 and SB202190. The biological consequence of p38 activation may vary under different conditions, although there is a multitude of evidence that it has anti-mitogenic activity. 1) p38 is most strongly activated by proinflammatory cytokines. 2) It has been show to inhibit cyclin D1 expression - a direct modulator of cellular proliferation, and 3) The p38 pathway activates heat shock protein-27 (Hsp27) *in vivo* which itself can inhibit cell proliferation (Chen *et al*, 2000).

1.6 The Ras Superfamily

The Ras Superfamily of small GTPases encompasses five subfamilies. There are: Rho-, ARF-, Rab-, Ran- and the Ras family itself, which together comprise more than 50 members. Small GTPases serve as crucial molecular switches in the regulation of intracellular signalling pathways (Exton, 1998). GTPases control biochemical events by cycling from the inactive GDP-bound state to active GTP-bound forms. This cycling is regulated by the opposing activities of guanine nucleotide exchange factors (GNEFs) and GTPase activating proteins (s) (Vojtek *et al*, 1998).

GNEFs (e.g. SOS, Vav) catalyse the activation of GTPases such as Ras by promoting the formation of active GTP-bound Ras, whereas GAPs (e.g. p120 GAP) accelerate the normally slow intrinsic GTPase activity of Ras to promote the formation of inactive GDP-bound Ras. The activated GTPase interact with specific target proteins that act as effectors to regulate downstream signalling cascades. See Figure 1.12.

Investigation into the roles of these proteins has been made easier with the use of dominant negative mutants, pharmacological inhibitors (e.g. farnesyltransferase inhibitors), and toxins. These bacterial products include the *Clostridium sordelli* lethal toxin (LT) - a high molecular weight toxin that covalently modifies small GTPases and has been used in several studies to highlight the contribution of Ras, Rap and Rac GTPases to cellular responses (Popoff *et al* 1996: Palsson *et al*, 2000).

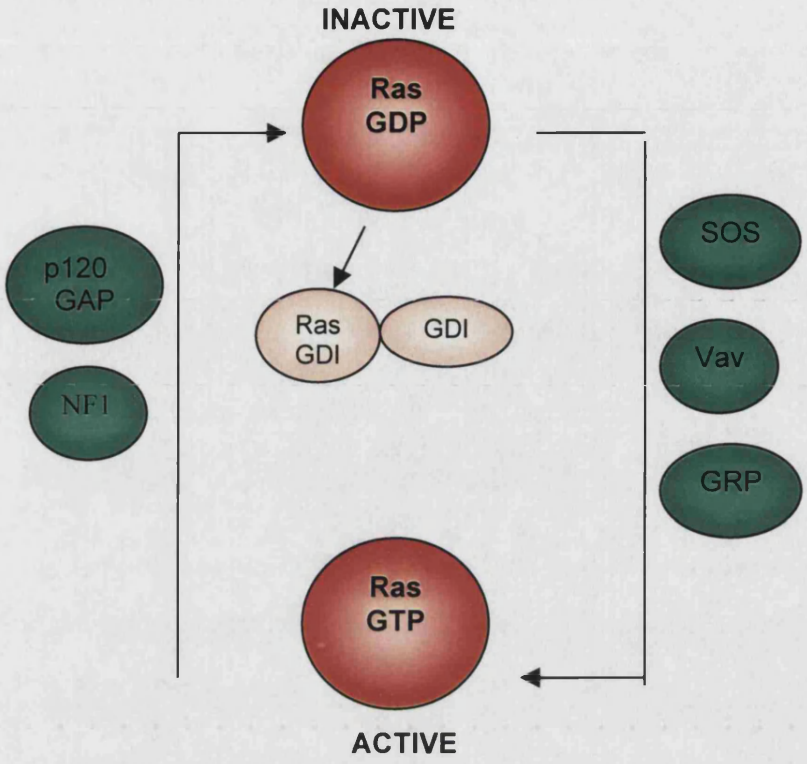


Figure 1.12 The GTPase cycle .Schematic view of the factors that regulate Ras. Proteins from the Ras family cycle between the active GTP-bound state and the inactive GDP-bound state. Guanine nucleotide exchange factors (GNEFs) catalyse the activation of Ras through promoting the dissociation of GDP, thus, allowing Ras to interact with target proteins to induce co-ordinated signals. Ras remains in this active state until the bound GTP is hydrolysed to GDP, a process accelerated by GTPase activating proteins (GAPs).

Rho GTPases

The Rho GTPase subfamily, which consists of the closely related proteins Rho, Rac and Cdc42, has been implicated in the regulation of a diverse range of cellular functions (Reviewed in Mackay and Hall, 1998; Reif and Cantrell, 1998). Many studies have established that Rho GTPases control cytoskeletal dynamics in many cell systems. Cdc42 induces the polymerisation of actin in neutrophils extracts (Benard *et al*, 1999), and Rho, Rac and Cdc42 have been implicated in the chemotactic response to chemokines in leukocytes, as well as its involvement in the phagocytic process (Del Pozo *et al*, 1999). Moreover, Rho, Rac and Cdc42 have been identified as key players in several leukocyte signalling pathways, such as in the activation of phospholipase D (Hammond *et al*, 1997) and PI3K (Parker, 1995). For example, both Rac and Cdc42 have been reported to associate with the

p85/p110 heterodimer (Tolias *et al*, 1995). Conversely, the cytoskeletal reorganisation induced by PI3Kγ overexpression was entirely dependent on Rac, but not Cdc42 (Metjian *et al*, 1998).

As well as cytoskeletal reorganisation, Rho proteins appear to trigger changes in gene transcription via the MAPK pathway (Figure 1.13). Several reports have implicated that both the JNK and Rac and Cdc42 can activate p38 MAP kinase cascades. This suggests an analogous role to that played by Ras in the MAPK pathway. We are still a long way from the biochemical description of the pathways linking active Rho proteins to cellular responses. However, the spectrum of their regulatory role is becoming wider. Already, Rho, Rac, Cdc42 have been implicated in cell movement, cytokinesis, axonal guidance, and morphogenetic processes involving changes in cell polarity and shape (Hall, 1998).

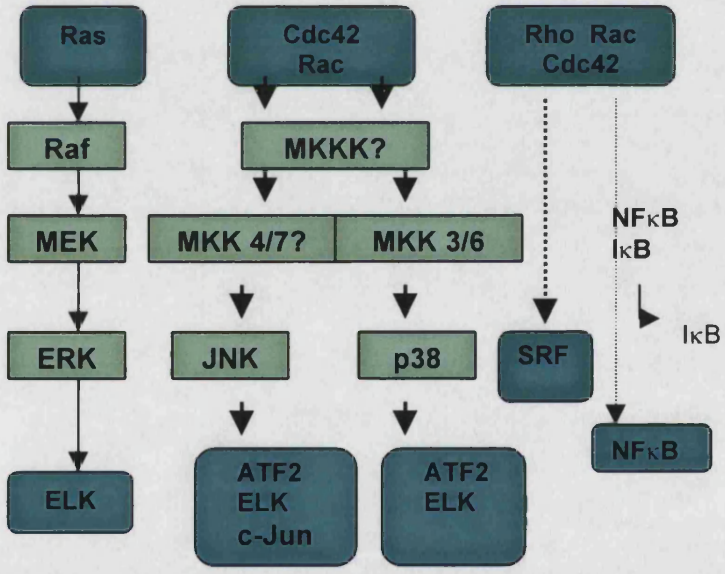


Fig 1.3. The role of Ras and Rho GTPases in gene transcription.
Ras is known to regulate the ERK pathway by directly activating Raf MAP kinase kinase kinase (MKKK). The regulation of JNK and p38 cascades and the subsequent activation of transcription factors appears to be more complex than that of the ERK pathway. Rac and Cdc42 can activate JNK and p38 MAP kinase pathways, although the mechanism is unclear. To date, eight MAP kinase kinase kinases have been described which are capable of activating JNK/p38, of these MKL, MEKK1 and MEKK4 interact directly with Rac and Cdc42. See text

Ras GTPase

The Ras family comprises H-Ras, K-Ras4A, K-Ras 4B, N-Ras, and other homologous proteins such as R-Ras, TC21, Rap and Ral. Ras proteins are proto-oncogene products that appear to be critical to signalling pathways spanning from the cell surface to the control of proliferation, differentiation or apoptosis (Rebollo and Martinez-A, 1999). Mutations in Ras at amino acids 12, 13 or 61 make Ras insensitive to GAPs, thus constitutively active. These mutations are extremely common in many human cancers, with an estimated 30% of all human tumours being due to the oncogenically activated Ras (Rommel and Hafen, 1998; Vojtek and Der, 1998).

Linking GPCRs and Gβγ to Ras.

There is a wealth of information documenting the Ras-MAPK pathways that are initiated by ligand-bound receptor tyrosine kinases (RTKs). Following the autophosphorylation and tyrosine phosphorylation of adaptor proteins like Shc and Grb2 guanine nucleotide exchange factors for Ras are recruited to a membrane-localised complex. More recently, the importance of GPCRs in both normal and aberrant growth control has become more evident, thus highlighting their key role in conveying mitogenic signals to the nucleus (Figure 1.14).

The inhibitory effect of genistein on LPA-induced MAPK activation was the first indication that tyrosine kinases may be involved the activation of MAPK by GPCRs (Hordijk *et al*, 1994). Additionally, many groups have demonstrated the rapid tyrosine phosphorylation of the SH2-containing protein (Shc) band and the subsequent formation of Shc-GRB2 complexes. This is a reaction in which several tyrosine kinases have been implicated, such as Fyn Lyn and Yes and the more distantly related Syk and Pyk2. The subsequent association of Shc with GRb2 serves to direct the guanine nucleotide exchanger, SOS, to the plasma membrane and into proximity with Ras (Wan *et al,* 1996).

For those GPCRs coupled to pertussis toxin (PTX)-sensitive G_i, such as lysophosphatidic acid (LPA), the pathway is initiated by the dissociation of the βγ subunits from the G protein complex (Heldin *et al*, 1999). This observation prompted the search for molecules acting downstream of Gβγ, and in many conditions, it was found that MAPK activation by βγ subunits did not require PLCβ or PKC activation.

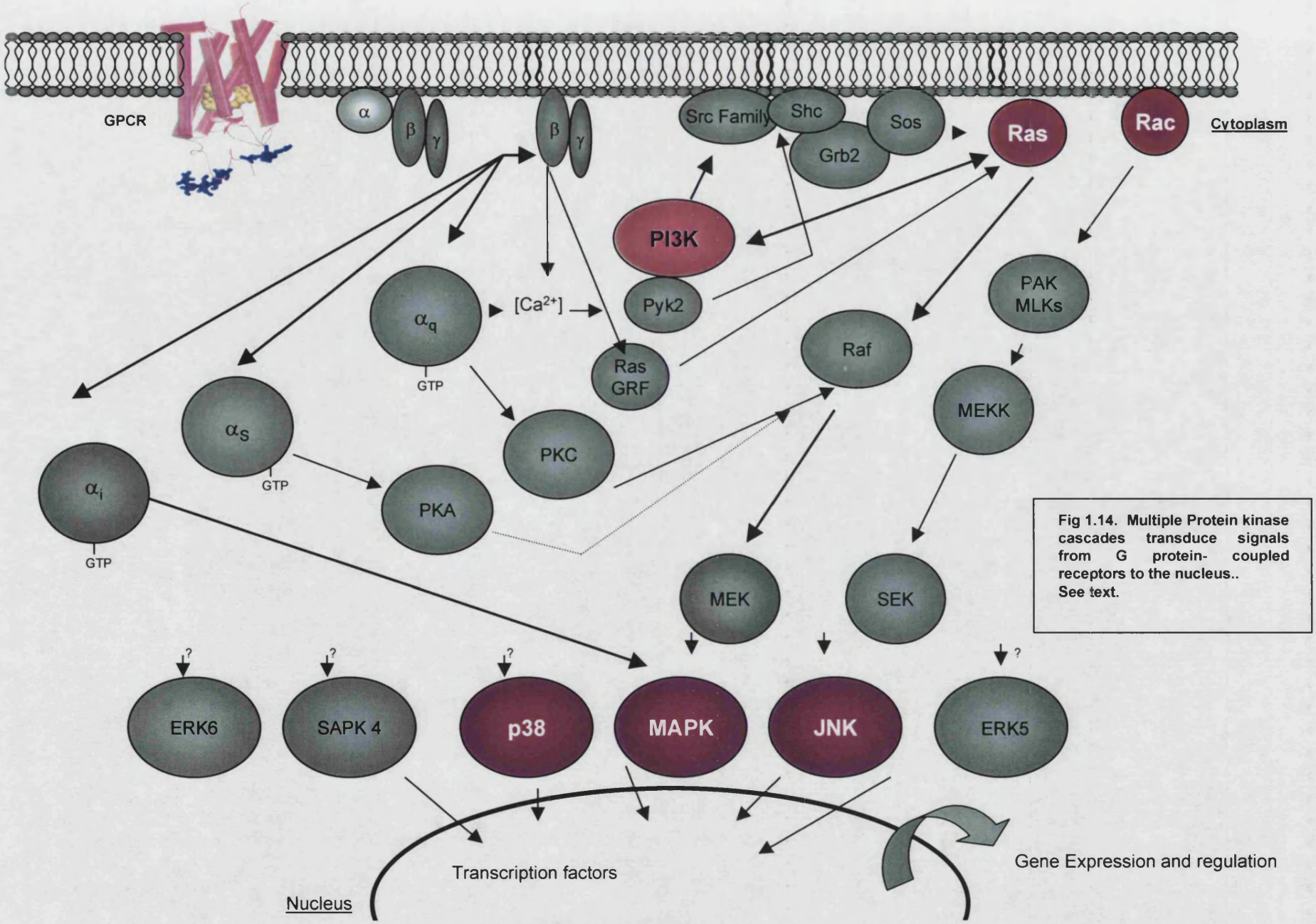


Fig 1.14. Multiple Protein kinase cascades transduce signals from G protein- coupled receptors to the nucleus.. See text.

Studies based on co-expression of Gβγ along with dominant negative mutants of Ras commonly resulted in the diminished activation of MAPK. (Della Rocca *et al*, 1997). Taken together, it was proposed that signalling from the GPCR to MAPK quite often functioned on Ras-dependent pathway.

The involvement of PI3K in the Ras/MAPK cascade has also been intensely investigated. Several groups have shown that by inhibiting PI3K, MAPK activation by GPCRs can be abrogated. For instance, PI3Kγ was found to function downstream of Gβγ but upstream of Src-like kinases – a potential mechanism whereby GPCRs modulate non-receptor tyrosine kinases (Hawes *et al,* 1996). Interestingly, there is now some evidence to suggest that there could well be Ras-independent routes for MAPK activation. Moreover, these pathways could potentially involve PI3Kγ. The molecular mechanisms by which this occurs are not well understood. One possible explanation for the PI3Kγ-mediated, Ras-independent pathway may involve the atypical ζ isoform of PKC (Takeda *et al*, 1999). This is further supported by the observation that direct stimulation of PKC by phorbol esters can activate MAPK in a Ras-dependent or Ras-independent manner.

It can be concluded here that multiple molecules are involved in the activation of MAPKs by GPCRs. The conflicting results obtained by several groups may be explained by the restricted tissue distribution of these molecules. The relative biochemical routes utilised to link GPCRs to the MAPK pathway may depend heavily on the repertoire of signalling molecules available in each a particular cell type.

Downstream Effectors of Ras

Ras mediates it effects on cellular proliferation primarily through the activation of a cascade of kinases. As previously discussed, the best-characterised downstream effector molecule of Ras is Raf (c-Raf-1, A-Raf and B-Raf), a serine/threonine kinase that links Ras to the MAP kinase cascade. The binding of Ras to Raf requires active, GTP-bound Ras, which is thought to occur via two distinct amino-terminal regions of Raf-1 (Vojdek and Der, 1998). It appears that Ras promotes not only membrane translocation of Raf, but may also facilitate the events leading up to its activation. In addition, other components appear to contribute to Raf-1 activation including 14-3-3 proteins, hsp90 and serine/threonine kinases indicating that the Ras-Raf connection is via a multicomplex of which the specific interactions remain to be determined.

Although extensive research has clearly implicated the involvement of PI3K in the Ras/Raf/MAPK cascade, it may also represent a direct target of active Ras in other pathways. PI3K has been shown to become activated by direct interaction of the catalytic sub-unit with Ras-GTP *in vitro* and *in vivo* (Kodaki *et al,* 1994). Several studies have demonstrated that levels of the phospholipid products of PI3K are elevated by the co-expression of active Ras and PI3K, while in the presence of Ras inhibitors; their production in response to stimulation is reduced. Furthermore, mutational studies in Ras imply a degree of co-operation between the Raf and PI3K pathways, in that they synergise strongly in transformation in the absence of Ras, indicating some biological responses initiated by Ras require the activity of both PI3K and Raf.

In addition to Raf and PI3K, other potential effectors of active Ras are the small G-proteins Ral and Rho. Until recently, the physiological relevance of their interaction with Ras was unknown. However, RalGDS has been found to interact with Ras and to function as an effector target in Ras signalling pathways, inducing cellular transformation in parallel with activation of the Raf/MAP kinase cascade (Reviewed in Wolthuis and Bos, 1999). Ral-A has been shown to be involved in tyrosine-kinase mediated activation of PLD, suggesting that the Ras-Ral pathway is linked to the regulation of phospholipid metabolism (Wang, 1999). Although Rho function has been shown to be required for Ras- mediated DNA synthesis, the available data regarding Rho as a Ras effector is limited.

1.7 A New Dimension in GPCR signalling - Chemokines

Despite the rapid advances in characterising new chemokines and their cognate receptors, the biochemical events leading to cell migration towards a chemotactic gradient remain ill understood. It is agreed that cell movement depends on multitude of tightly orchestrated cellular processes such cytoskeletal reorganisation, alterations in integrin affinity and integrin cycling. The signalling cascades pertaining to these events are only just beginning to emerge, and these observations are, in many cases comparable to those observed in other GPCR systems.

Stimulation of chemotaxis requires the functional coupling of the receptor to G proteins. It is here that chemokine-stimulated signal transduction is initiated. On the whole, chemokine receptors are usually coupled to $G\alpha_i$ proteins that render cellular responses sensitive to pertussis toxin treatment. However, SDF-1, and RANTES induce coupling to pathways that are both sensitive (G_i) and insensitive (G_q) to pertussis toxin (Bacon *et al,* 1995; Bleul *et al*, 1996). Interestingly, splice variants of MCP-1 receptor, CCR2, exhibits a unique specificity in its coupling to the α subunits of the G_q class of G proteins

Appropriate stimulation of a chemokine receptors rapidly activates PLC-β_2 and PLC-β_3 isoenzymes which ultimately leads to $InsP_3$ generation and a transient rise in calcium concentration. The activation of the PLC-β isoforms by $G\beta\gamma$ subunits is well-established (Schall and Bacon, 1994; Baggiolini, 1997). In PLC-$\beta_{2/3}$ knockout mice, chemokine-stimulated calcium transients are completely abrogated (Li *et al*, 2000) – another confirmation that these are the isoforms of choice in chemokine-activated leukocytes.

Another well-documented effector of $\beta\gamma$ subunits is the G protein-specific PI3K, PI3Kγ, and studies from gene-targeted mice have demonstrated its relative contribution in chemokine-mediated cell function. PI3Kγ knockouts have severely impaired ability in chemokine signalling, which is good indication that this isoform is an essential element in pathways downstream of some GPCRs.

The recently reported activation of the Src-family kinases by $G\alpha$ subunits and RGS (regulators of G protein signalling) has refreshed an interest in subunit-regulated signalling (Morris and Malbon, 1999). Although we are awaiting direct confirmation from chemokine systems, $G\alpha_i$-mediated signalling downstream of these receptors is assumed from inference and indirect evidence. There is considerable structural similarities between the kinase domain (SH1) among Src-family enzymes, so it reasonable to assume that $G\alpha_i$ subunits could also activate other Src-related kinases with leukocytes, such as Fgr, Lck or Lyn (Ganju *et al*, 1998; Blaukat *et al*, 1999; Wang *et al*, 2000). This would not only account for the linkage of chemokine receptors to the Ras-MAPK cascade via the "classical" tyrosine kinase pathway, but may also explain the activation of Focal adhesion kinase (FAK) and Pyk-2 by chemokines (Wang *et al*, 2000; Y Sotsios, unpublished data, Yamasaki *et al*, 2001).

Figure 1.15 ***Chemokine Signalling***

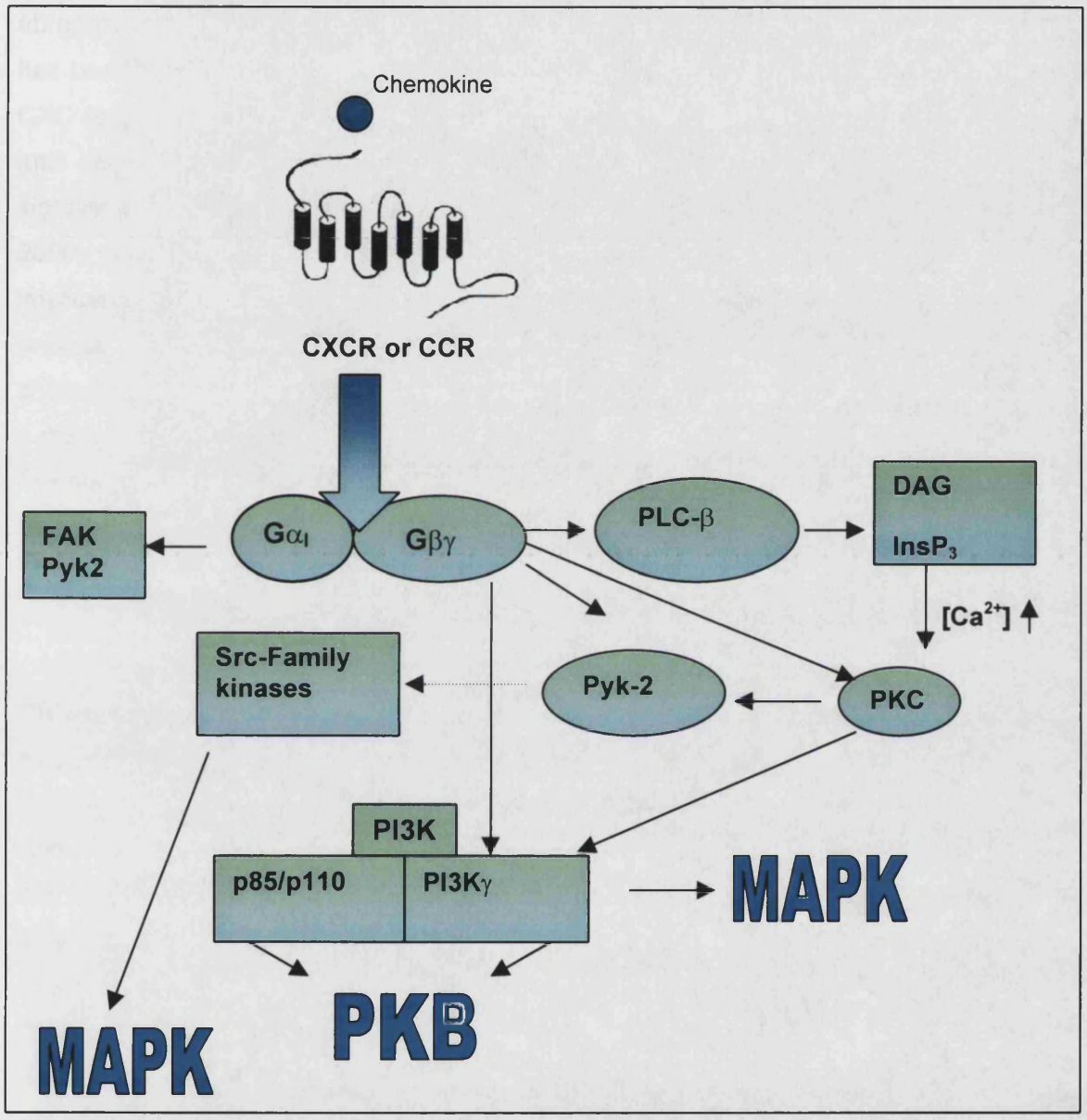


Fig 1.15 Chemokine signalling ensuing receptor ligation. Receptor ligation instigates the release of βγ subunits from $G\alpha_i$- and G_i-coupled receptors. Gβγ rapidly activate PLC and consequently hydrolyses PtdIns(4,5)P_2 to form DAG, and release calcium from intracellular stores through generation of IP_3. GPCRs have been reported to activate MAPK through βγ-activated PI3Kγ in both PKCξ-dependent and –independent means. Activation of Pyk2 by βγ subunits is through an indirect mechanism – quite possibly via a βγ-mediated rise in $[Ca^{2+}]$ and PKC activation leading to a transient association between Pyk2 and Src (Blaukat *et al*, 1999). In contrast, $G\alpha_i$ could stimulate phosphorylation of Src-family kinases directly, and activate MAPK via the "classical" (Shc/Grb2/SOS) pathway. Activation of both Class I PI3Ks (p85/p110 and PI3Kγ) may lead to protracted PtdIns(3,4,5)P_3 accumulation and subsequent phosphorylation of PKB. This scheme, which is not complete, highlights the key effectors following stimulation of a hypothetical generic chemokine receptor. For simplicity, the potential signalling events between the pathways are omitted – see text for detailed account.

PI3-kinase: A Role in Chemokine Signal Transduction?

The very first report that PI3K was involved in chemokine-stimulated cells was that the polarisation and chemotaxis of T cells mediated by RANTES/CCR5 could be abrogated with pharmacological inhibitors of the enzyme (Turner *et al*, 1995). This has been reiterated for other CC chemokines such as MCP-1 and IP-10, as well as CXC (e.g. SDF) (Turner *et al* 1998; Bonacchi *et al*; 2001). It is clear that production and degradation of 3-phosphorylated lipids is an important event in developing signalling gradients and subsequent cell migration (reviewed in Sotsios and Ward 2000; Curnock *et al*, 2002). Both Class $I_{A/B}$ and Class II PI3-kinases have been implicated in biochemical and functional roles in several chemokine systems, but the precise role of each isoform are not completely understood. The diversity of chemokine receptors to couple to different G proteins and PI3K isoforms, is perhaps a means in which these receptors can simultaneously exert control over a number of functional events (e.g. polarisation, superoxide release, lamellipodia formation) as well as transcriptional and post-translational control of the cell.

Phospholipase D – a mediator in Chemokine Signalling?

As previously discussed, several studies have reported phospholipase D (PLD) to be activated by several GPCRs including IL-8 and RANTES chemokine receptors (Bacon *et al*, 1995,1999). The possible involvement of PI3K and ARF proteins – has implications for our understanding of PLD regulation and actin polymerisation by chemokines. So, given the evidence, it is fair to assume that chemokines induce migration through its regulation of actin polymerisation. This is achieved (in part) through PtdIns(3,4,5)P_3–modulation of ADP-ribosylation factors (ARFs), such as ARF-6, which subsequently leads to activation of PLD. This is of particular interest since PI3Kγ has been shown to play a key role on reorganisation of the cytoskeleton (Metjian *et al*, 1998).

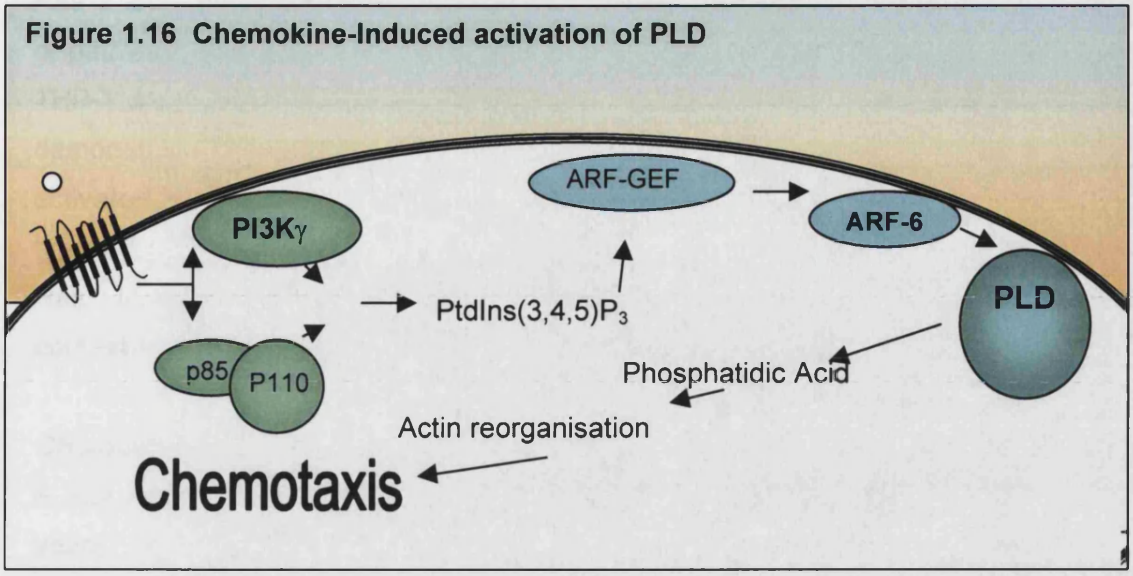


Figure 1.16 Chemokine-induced activation of PLD is facilitated by PI3K and ARF proteins. $PtdIns(3,4,5)P_3$ is increased in response to chemokine stimulation via PI3Kγ (or p85/p110 heterodimer) and promotes recruitment of PH domain containing ARF-GEFs (e.g. ARNO) to the cell membrane. Here, the ARF-6 is cycled into its active GTP-bound state and subsequently activates PLD. The resultant accumulation of phosphatidic acid is thought to mediate cytoskeletal reorganisation and shape change – structural pre-requisites for cell migration. See text.

The Ras superfamily – Molecular Switches for chemokine signalling

The structural reorganisation of the cytoskeleton including compartmentalisation and cell polarisation is a prerequisite for chemotaxis. Members of the Rho family of GTPases, namely Rho, Rac and Cdc42 are pivotal in deploying such events,. For this reason, much interest has focussed on the role that these GTPases play in both cell migration and its involvement in the PI3K signalling system (Metjian *et al*, 1998; MacKay and Hall, 1998; Jimenez *et al*, 2000).

A role for Cdc42 in chemotaxis has been demonstrated using CSF-1-stimulated macrophages injected with the dominant negative N17Cdc42 (Allen *et al*, 1998; Haddad *et al*, 2001). Moreover, in leukaemic T cell lines, these mutants display a much more inhibitory effect towards a chemotactic gradient than dominant negative mutants of RhoA and Rac (Del Pozo *et* al, 1999). It appears that Cdc42 and its interaction with downstream effectors, such as Wiskott-Aldrich syndrome protein (WASP) and p21-activated kinases (PAKs), plays a crucial role in the directional migration of leukocytes.

Ras is a versatile intracellular switch that has been implicated in only a small number of pathways activated by chemokines. Both MCP-1 and SDF-1 can activate Ras in THP-1 and Jurkat cells respectively (unpublished observations) whilst IL-8 has been demonstrated to activate Ras in neutrophils. The mechanisms by which Ras is activated are not well understood, although stimulation of other GPCRs results in tyrosine phosphorylation events that ultimately lead to recruitment and activation of Ras. In most cases, chemokine-stimulated Ras activation has been studied in the context its interaction with the PI3-kinase and MAPK pathways.

Chemokines Stimulate the MAPK cascade

A role for the MAPK cascade in cell motility has been recognised for a number of years. Amoeboid chemotaxis of fibronectin-stimulated fibroblasts was one of the first indications that ERK was involved in this process. Given the surge of interest over the past 5 years in chemokine signalling events, characterisation of the chemokine-induced MAPK (including ERK, p38 and JNK) activation has been well documented. This has been mainly achieved by the inhibition of ERK activation with MAP kinase (MEK) inhibitors such as PD98059 that has abrogated chemotaxis in response to several chemokines. Such experiments have shown that ERK activation is necessary for SDF-1 and MIP-3α induced migration (Sullivan *et al*, 1999; Tilton *et al*, 2000). The molecular mechanism of ERK activation in response to chemokines remains a matter of contention.

Aims and Objectives

Given the recent surge of interest in leukocyte migration in response to chemokine stimulation, here I attempt to investigate the intracellular signalling events underlying this process. Although the role of pivotal enzymes such as PI3-kinase and MAPK, and their regulation has been characterised in a number systems, their role in cell migration remains poorly understood. In this thesis, the objective is to assess the ability of MCP-1, MCP-2, MCP-3 and MCP-4 to stimulate signal transduction in the monocytic cell line, THP-1, and to characterise the nature of these interactions. Once a clear biochemical profile has been achieved, I aim to link these events functional output

Insofar as resources and time permits, this study aims to:

- Compare and contrast the signal transduction profiles of MCP-2, -3 and –4, to that of the more extensively characterised MCP-1.

- Assess the role of PI3-K in other MCP-induced signalling pathways and the subsequent consequences on cell migration.

- Demonstrate any differences in activation, kinetic profile and functional significance of the various PI3K isoforms.

- Attempt to extrapolate these data to other chemokine systems and suggest how the exploitation of molecular targets could be applied to the pathophysiology of atherosclerosis and other inflammatory disorders.

Figure 3.1 *R*T-PCR analysis of CC chemokine receptor expression.

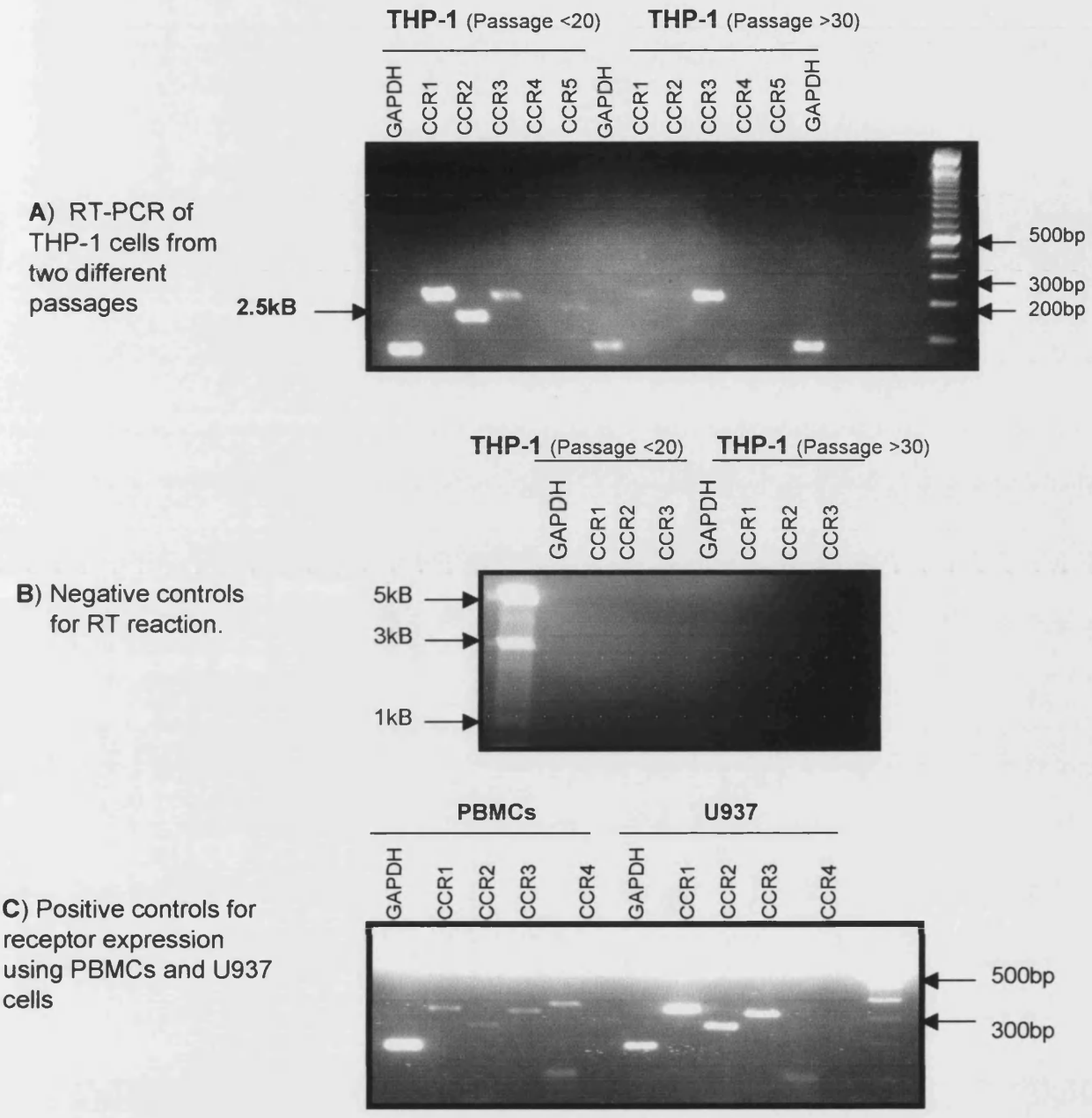


Figure 3.1 *R*T-PCR analysis of CC chemokine receptor expression. 1*ug* of DNAase I-treated mRNA was used per reaction and identical reactions were carried out for each cell line. PCR was performed for 30 cycles and the oligonucleotide primers used are under 'Materials and Methods'. In **A**, THP-1 cells from different passage numbers (1-20 and 30+), and over were screened for the CC chemokine receptors CCR1, -2, -3, -4, and 5. As a negative control (**B**), both cell lines were screened for GAPDH CCR1, -2, and -3 in the absence of reverse transcriptase. (**C**). Peripheral blood mononuclear cells and the U937 cell line was used as a positive control for CCR1, -2, -3 and 4. 10µl from the final reaction mixture was electrophoresed on a 2% agarose, 1 xTAE at 150V for 90 min and the DNA visualised by ethidium bromide staining of the gel. The sizes of the DNA molecular weight markers (base pairs) are shown in the lanes next to the samples.

Figure 3.2 Surface expression of CCR2 on unstimulated THP-1 cells.

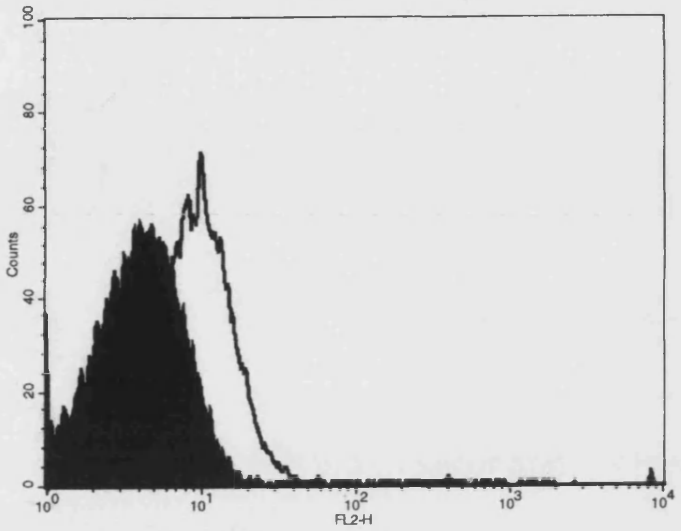
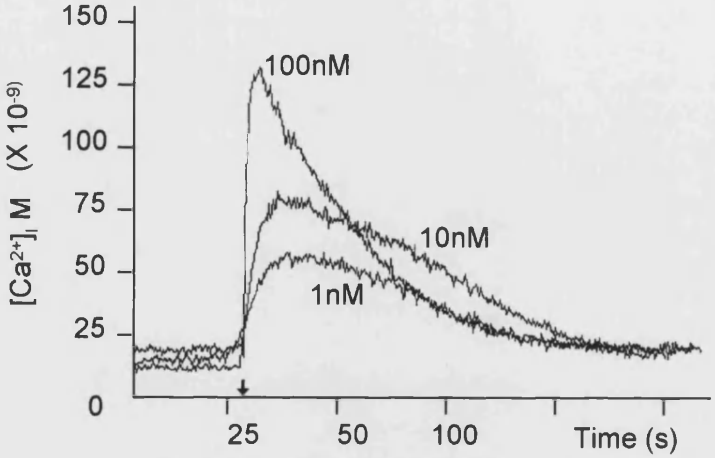
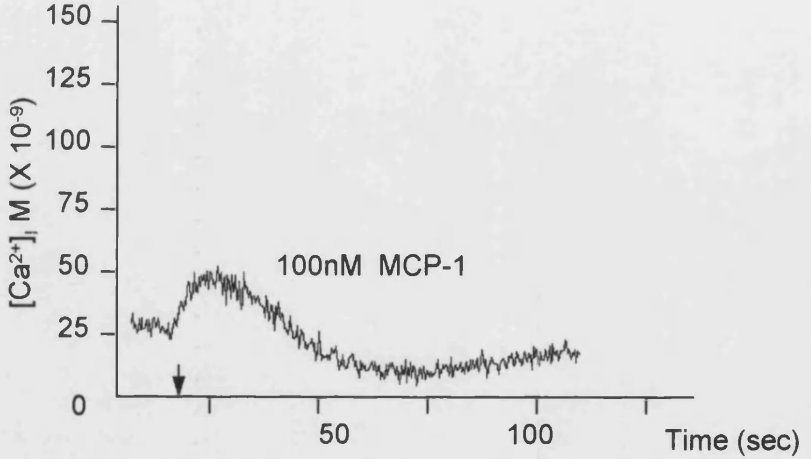


Figure 3.2 Surface expression of CCR2 on unstimulated THP-1 cells. 1 x 10^5 THP-1 cells per point were analysed for surface expression of CCR2, with FITC-conjugated mouse $IgG2_B$ anti-human antibodies (transparent histogram), or isotype matched control antibodies (black histogram). The cells were analysed on a Becton Dickinson FACS Vantage as described in 'Materials and Methods'. The expression profile shown is from one experiment, but is typical of CCR2 expression profiles on THP-1 cells used in all other experiments.

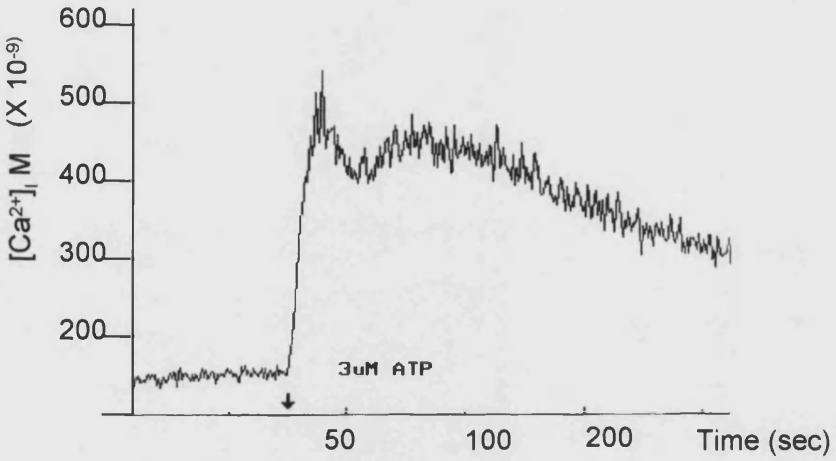
Figure 3.3 Effect of MCP-1 on calcium mobilisation in THP-1 cells



***A*) MCP-1 stimulated THP-1 cells (dose-response).**



***B*) MCP-1-stimulated THP-1 cells in the absence of extracellular calcium.**



C) ATP-stimulated THP-1 cells
Positive control for calcium mobilisation

Figure 3.3 Detection of Intracellular [Ca^{2+}] in THP-1 cells. 2 x10^6 cells were loaded with Fura-2AM and assayed at 37°C in a continuously stirring cuvette in a Photon Technologies fluorimeter(nm), (340 nm{λ_{ex}}; 380nm {λ_{em}}), with fluorescence emission recorded every 100 ms for at least 180 s. All experiments were conducted in the presence of extracellular calcium as indicated under "Materials and Methods" unless otherwise stated. MCP-1 was added at the times indicated (⬇). In **A**, MCP-1 was added to final concentrations of 1, 10 and 100nM. Graph represents three separate stimulations superimposed onto one axis. **B.** Cells were stimulated with 100nM MCP-1 in the absence of extracellular calcium. **C**. As a positive indicator of calcium mobilisation, cells were stimulated with ATP (3μM). Traces are in M [Ca^{2+}] from one experiment representative of at least 4 other independent separate experiments.

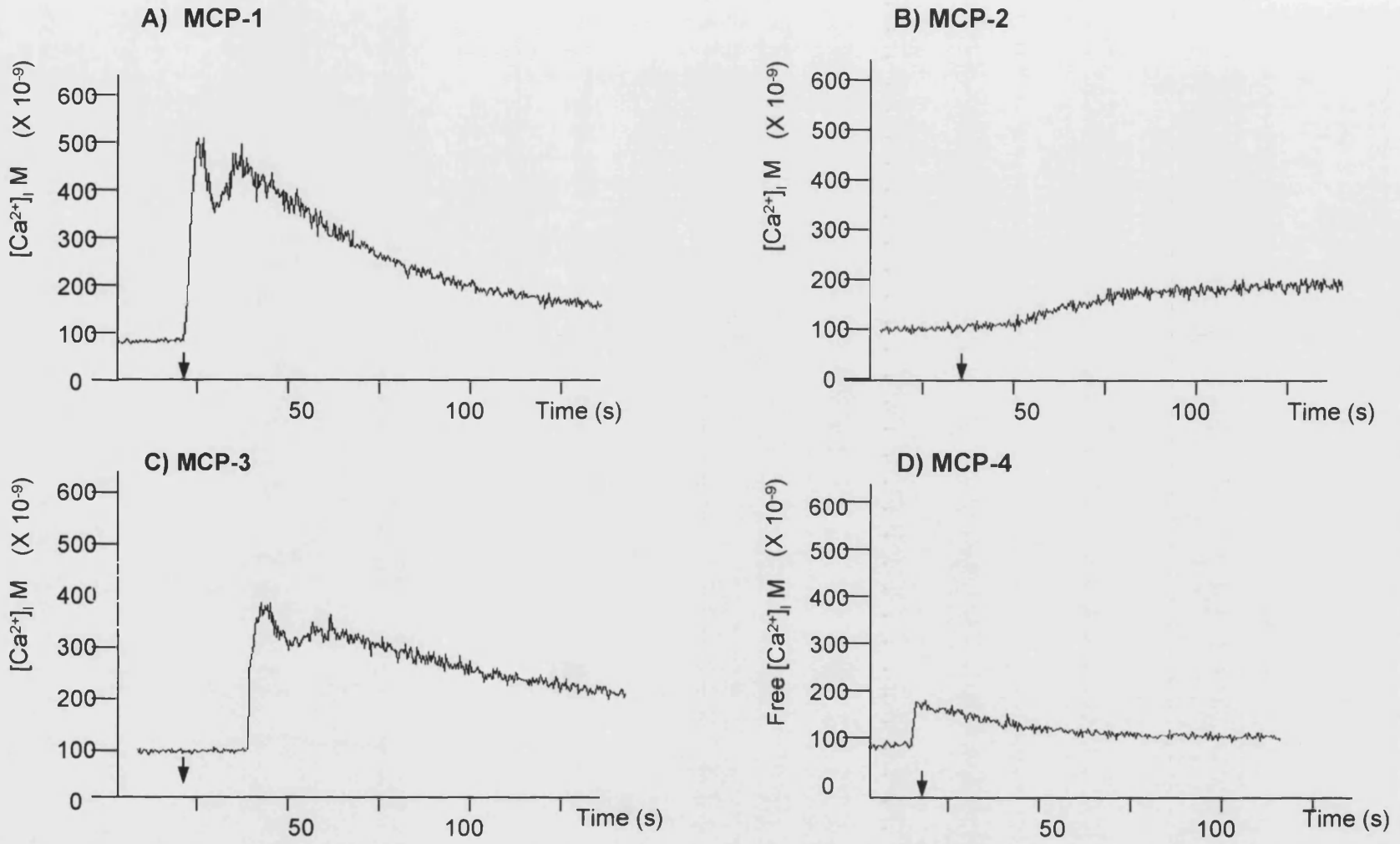


Figure 3.4 Comparison of MCP-1, -2, -3, and –4-Induced calcium $[Ca^{2+}]_i$ in THP-1 cells. 2 x10^6 cells were loaded with Fura-2AM and assayed at 37°C in a continuously stirring cuvette in a Photon Technologies fluorimeter (340 nm{λ_{ex}}; 380nm {λ_{em}}), with fluorescence emission recorded every 100 ms for at least 180 s. All experiments were conducted in the presence of extracellular calcium as indicated under 'Materials and Methods' unless otherwise stated. Cells were treated with 100nM of MCP-1, -2, -3, and –4 in **A**, **B**, **C** and **D**, respectively.

Figure 3.5A. Induction of THP-1 cell migration in response to MCP-1 and MCP-2

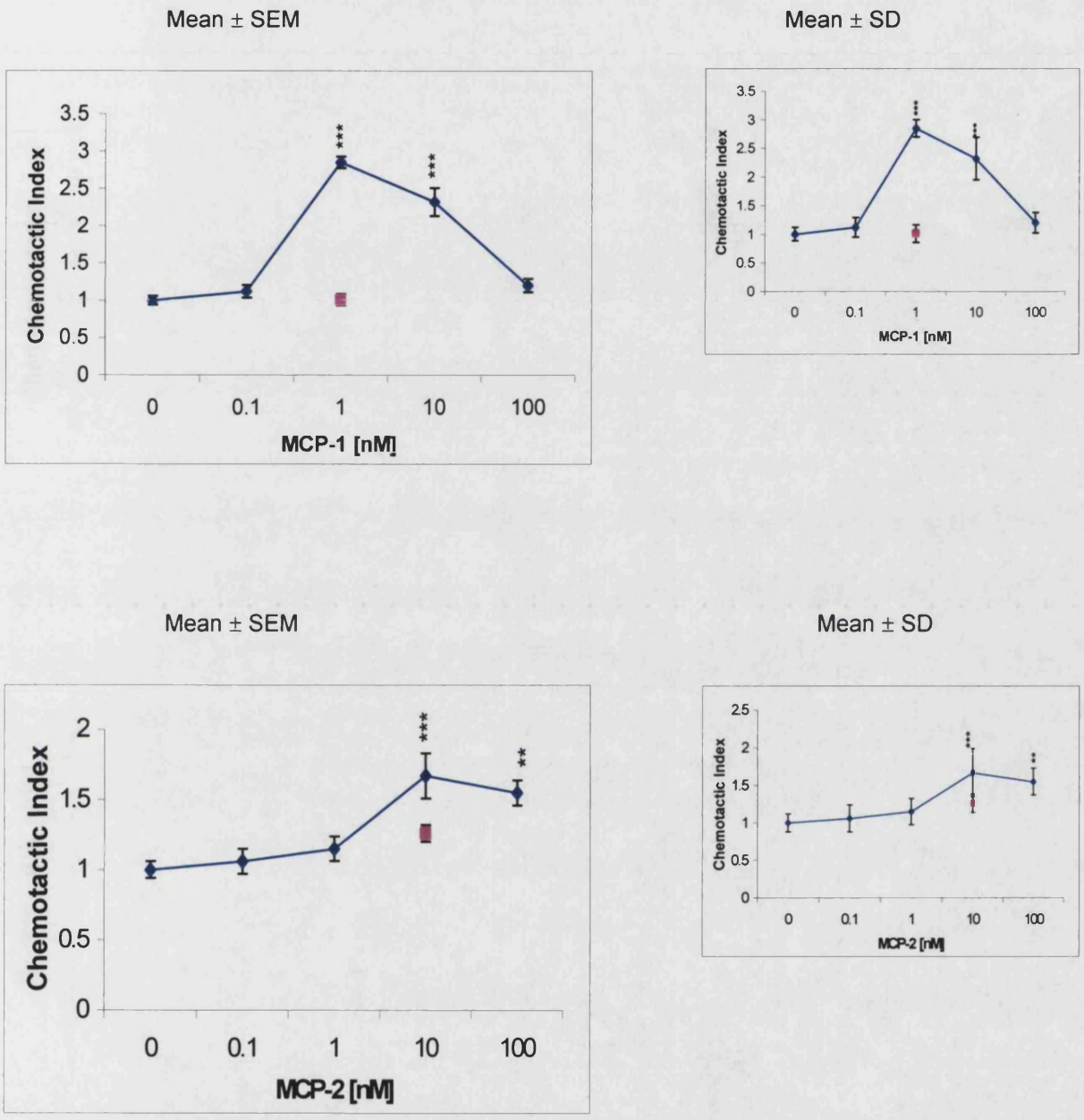


Figure 3.5A The Induction of cell migration by MCP-1 and MCP-2 in THP-1 cells. 1 x 10^5 THP-1 cells per point were assayed for chemotactic ability in a 96-well Neuroprobe chemotaxis chamber. Cells were left to migrate for 3 hours to either 0.1% BSA (control), or various concentrations of chemokine as indicated. Results are expressed as a chemotactic index (C:I): the ratio of stimulated over basal migration. For each experiment a 'non-directed' chemokine gradient was also established (chemokine in both top and bottom wells) to control for chemokinetic migration. Data represent the mean ± SEM (5 replicates per chamber/2 chambers per experiment). For comparative reasons, graphs show calculated mean ± SEM (left) and mean ± SD (right). 5 replicates per chamber/2 chambers per experiment. Results are from one experiment but are representative of at least three others. *:p<0.05, **:p<0.01, ***p<0.001.

Figure 3.5B. Induction of THP-1 cell migration in response to MCP-3 and MCP-4

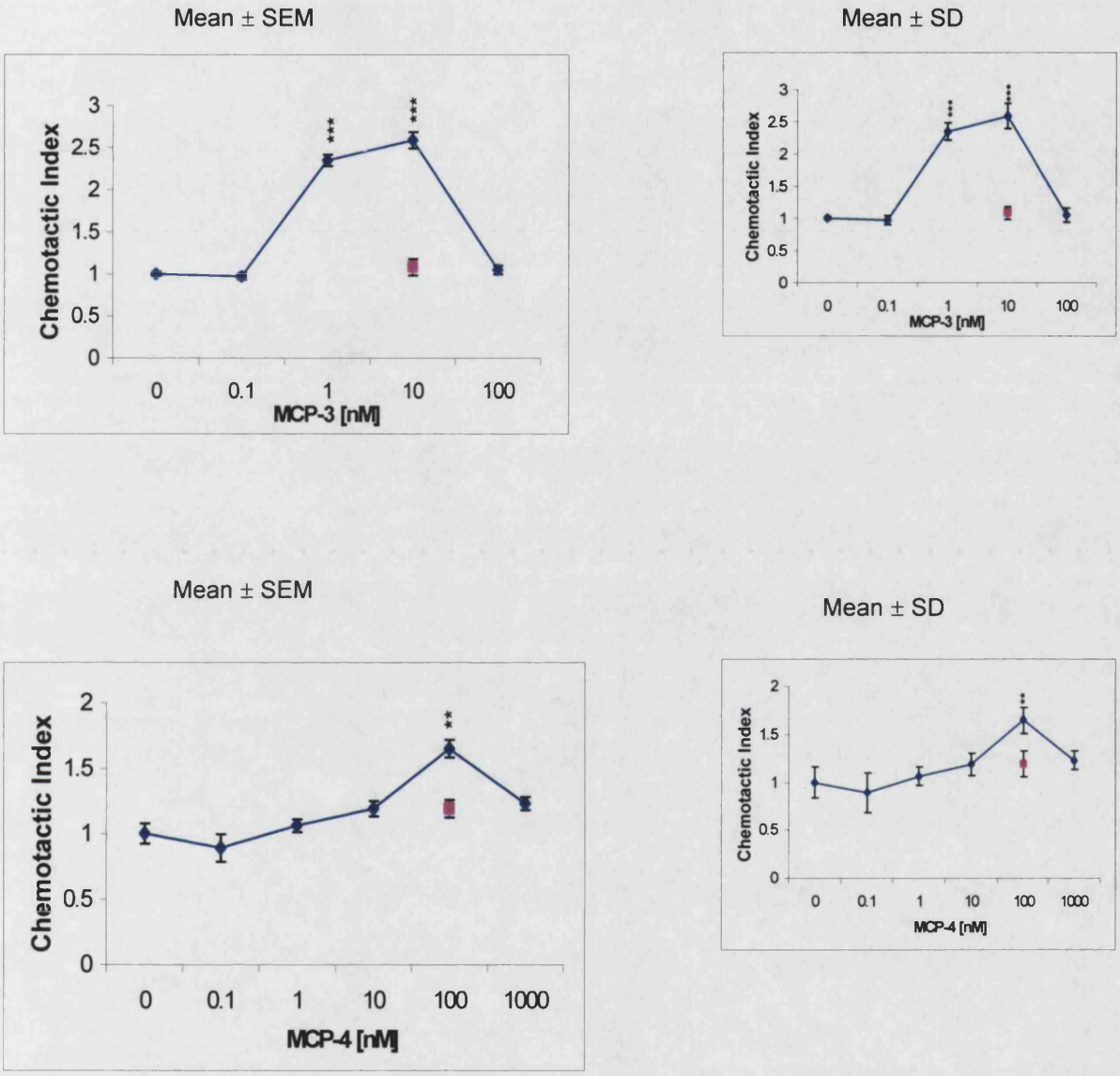


Figure 3.5B The Induction of cell migration by MCP-3 and MCP-4 in THP-1 cells. 1 x 10^5 THP-1 cells per point were assayed for chemotactic ability in a 96-well Neuroprobe chemotaxis chamber. Cells were left to migrate for 3 hours to either 0.1% BSA (control), or various concentrations of chemokine as indicated. Results are expressed as a chemotactic index (C:I): the ratio of stimulated over basal migration. For each experiment a 'non-directed' chemokine gradient was also established (chemokine in both top and bottom wells) to control for chemokinetic migration. For comparative reasons, graphs show calculated mean ± SEM (left) and mean ± SD (right). 5 replicates per chamber/2 chambers per experiment. Results are from one experiment but are representative of at least three others. *:p<0.05, **:p<0.01, ***p<0.001.

Figure 3.5C Effect of CCR2 ligands on THP-1 cell chemotaxis

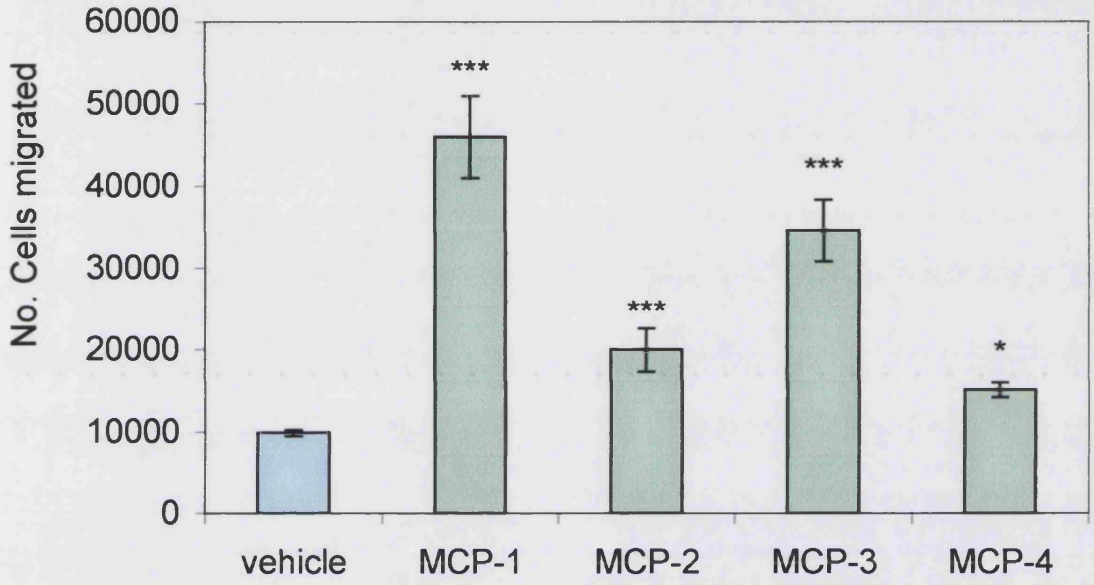


Figure 3.5C. Effect of CCR2 ligands on THP-1 cell chemotaxis. 1 x 10^5 THP-1 per point were assayed for chemotactic ability in a 96-well Neuroprobe™ chemotaxis chamber. Cells were left to migrate for 3 hours towards either vehicle (0.1% BSA), MCP-1 (1nM), MCP-2 (10nM), MCP-3 (10nM) or MCP-4 (100nM). Migration was determined as described in 'Materials and Methods'. Data represent the mean ± SEM (5 replicates per chamber/2 chambers per experiment). Results from one experiment but are representative of two other independent experiments. *:$p<0.05$, **:$p<0.01$, ***$p<0.001$.

Figure 3.6 The relative contribution of CCR2 to MCP-1, -2, -3 and -4-induced signal transduction in THP-1 cells

% inhibition of MCP-stimulated phospho-ERK-1/2 by SB282241

MCP-1	98%
MCP-2	82%
MCP-3	71%
MCP-4	82%

NB* Consideration was given to the fact that the basal activity of ERK-1/2 did fluctuated between experiments. Results indicate the degree of inhibition by the SB282241 compound between vehicle control and 2 m post-stimulation.

Figure 3.6 The relative contribution of CCR2 to MCP-induced signalling in THP-1 cells. THP-1 cells (1 x10^6 per point), untreated or preincubated for 15 minutes with 1μM SB282241, were exposed to the indicated time courses to MCP-1, MCP-2, MCP-3 and MCP-4 (1nM, 100nM, 10nM, and 100nM, respectively). Total cell lysates (2.5 x 10^5 cell equivalents per lane) were were resolved separately by SDS-PAGE, electrophoretically transferred to a nitrocellulose membrane and immunoblotted with anti-active ERK antibodies. A corresponding set of lysates were probed with anti-inactive ERK antibody to ensure equal loading of proteins. The results are representative of two experiments. Table shows percentage inhibition of MCP-stimulated ERK activity by SB282241 compared to vehicle (as assessed by densitometric analysis).

Section 4: Results

CCR2 ligands activate PI3-kinase signalling pathway

Rationale

The term 'PI 3-kinase' (PI3K) is now applied to a growing family of proteins that are able to convert PtdIns, PtdIns(4)P and PtdIns(4,5)P_2 into PtdIns(3)P, PtdIns(3,4) and PtdIns(3,4,5)P_3, respectively by phosphorylating the D-3 position of the inositol heads of D-3 phosphoinositide lipids. PI3K pathways have for some time been regarded as important intracellular signals that are upstream of a variety of responses, and more recently have been the focus of much attention with respect to their role in chemokine mediated functional responses.

There were two main procedures used in this study for the measurement of PI3-kinase activity, both of which were based on the detection of the transfer of the γ-phosphate of ATP to the D-3 position on the inositol ring. The first method relies on the metabolic labelling of intact cellular pools of ATP with [^{32}P]P_i followed by lipid extraction, and the measurement of lipid kinase activity by HPLC analysis. In the second procedure, assays of specific immunoprecipitated PI3-kinase isoforms were assessed for associated lipid kinase activity.

Measurement of D-3 Phosphoinositide Lipids

Analysis of the deacylated phospholipids lipids was achieved by HPLC, and the resulting reproducible chromatographic traces enabled the detection and quantification of the fluctuations in glycerophosphoryl derivatives (GroPtdIns lipids). Products of interest, namely PtdIns(3,4,)P_2 and PtdIns(3,4,5)P_3 were identified from retention times obtained with tritium-labelled phosphoinositide lipid standards. Increases in PtdIns(3)P were not detectable in THP-1 cells as resolution of this lipid was poor. Figure 4.1 shows a representative HPLC trace of unstimulated vs. CD3-stimulated Jurkat cells. This treatment commonly served as a positive control for [^{32}P] loading and as a standard for ligand-induced D-3-phosphoinositol lipid formation. Experiments showed that despite the consistency in relative accumulation of GroPtdIns lipids, loading efficiencies of [^{32}P] into THP-1 cells could not be accurately standardised. Hence, for objective comparison some results are expressed as a percentage increase from baseline, whereby the maximum increases ranged from 150 to 700 percent from basal PtdIns(3,4,5)P_3 levels. Although relative

kinetic profiles of D-3-lipid generation were consistent, time points for peak responses sometimes varied between experiments by up to 60 seconds. The time-courses shown are representative of at least four separate experiments

Accumulation of D-3-lipids induced by MCP-1 in THP-1 cells

The THP-1 cell line is known to have some constitutive PI3-kinase activity (Turner *et al*, 1998) and this was reflected in the basal generation of PtdIns(3,4)P_2 and PtdIns(3,4,5)P_3 in the unstimulated control (1835 and 1798 cpm, respectively). Stimulation with MCP-1 resulted in a rapid and transient increase in PtdIns(3,4,5)P_3 that was dependent on the concentration of MCP-1 (Figure 4.2). Maximal accumulation of this lipid (3128 cpm) was observed at 60 seconds post-stimulation, with an optimal concentration of 60nM MCP-1. Similarly, the optimum time point for accumulation of PtdIns(3,4,)P_2 was also after 60 seconds after ligation, yet kinetics appeared to be slightly slower and more sustained than that observed for PtdIns(3,4,5)P_3. Maximum accumulation of PtdIns(3,4)P_2 (3646 cpm) was attained at the higher concentration of 180nM MCP-1.

As several isoforms of PI3-kinase have been identified, it was necessary to determine which of these were responsible for the increases in D-3-lipid generation following MCP-1 stimulation. The first step taken to characterising these isoforms was through testing the sensitivity of D-3-lipid accumulation to pharmacological inhibitors, LY-290042 and pertussis toxin. Treatment with PI3-K inhibitor, LY-290042 (10μM), appeared to have no effect on MCP-1-induced accumulation of PtdIns(3,4,5)P_3 or PtdIns(3,4)P_2 (Figure 4.3) whereas pre-treatment with G protein inhibitor, pertussis toxin (100ng/ml), completely abrogated the MCP-1-induced increase in PtdIns(3,4,5)P_3 to below baseline levels (Figure 4.4). The effects of pertussis toxin on PtdIns(3,4)P_2 generation were not assessed.

Effects of MCP-2, -3 and -4 on D-3-lipid accumulation in THP-1 cells

MCP-2 stimulated THP-1 cells also resulted in an increase of PtdIns(3,4,5)P_3 and PtdIns(3,4)P_2. The elevation of both lipids exhibited bell-shaped characteristics with the maximum response observed in the presence of 60nM MCP-2 (an increase of 888 and 850 cpm above basal, respectively). (Figure 4.5). Furthermore, MCP-2-stimulated formation of PtdIns(3,4,5)P_3 and PtdIns(3,4)P_2 was rapid and transient since they were detectable 10 s after stimulation and peaked after 60 s. The observed responses were refractory PI3-K inhibition with LY-290042 (Figure 4.6).

MCP-3 induced an increase in PtdIns(3,4,5)P_3 which was optimal at 60nM MCP-3 (Figure 4.7). This transient response peaked at 60 s and had returned to near basal levels 2 m after stimulation. A rapid accumulation of PtdIns(3,4,5)P_3 was achieved using 180nM MCP-3, however, this increase was short lived, and had returned to baseline after only 30 s. With regards to formation of PtdIns(3,4)P_2, a concentration-dependent increase was also observed. A rapid increase in this lipid was achieved 30 s post-MCP-3 stimulation (180nM). . Pre-treatment with LY-290042 had no affect on the generation of PtdIns(3,4,5)P_3 nor PtdIns(3,4)P_2 (Figure 4.8).
Figure 4.9 shows the elevation of PtdIns(3,4,5)P_3 and PtdIns(3,4)P_2 in response to MCP-4. There was a transient elevation in both products that increased up to 60 s post-MCP-4 stimulation. The response was also concentration-dependent in that maximum accumulation of both PtdIns(3,4)P_2 and PtdIns(3,4,5)P_3 was obtained using 180nM MCP-4. As expected, neither of the lipids were affected by pre-treatment with LY-290042 (Figure 4.10).

Although MCPs 1-4 have all been shown to signal via CCR2, it appears that they induce signal transduction in THP-1 cells with distinct levels of efficacy. Figure 4.11 shows the relative potencies of these CCR2 ligands on PtdIns(3,4,5)P_3 accumulation in THP-1 cells. By stimulating THP-1 cells with concentrations of MCP-1-4 known to elicit an optimal response (60nM), a direct comparison of these chemokines could be made. The maximum response was observed with MCP-3 which increased PtdIns(3,4,5)P_3 generation to approximately 500% of basal level. This was then followed by MCP-1 (300%), MCP-2 (190%) and MCP-4 (170%). In terms of rank order of potency, it transpires that MCP-3>MCP-1>MCP-4>MCP-2 in the generation of PtdIns(3,4,5)P_3.

Activation of PKB by CCR2 ligands

Protein kinase B (PKB) is a proto-oncogene that is activated in signalling pathways initiated by PI3-kinases. It is thought to be recruited by and dependent on the phospholipid products of PI3-K for subsequent phosphorylation and activation. To support the observed CCR2-mediated, G-protein-dependent PtdIns(3,4,5)P_3 formation, the activation of PKB (a major downstream effector of PI3K) was assessed. This was achieved by western blotting THP-1 cell lysates with a phospho-specific antibody against Ser^{473} within the PKB activation loop - a residue implicated in activation of PKB *in vivo*. The principle-characterised substrate of PKB is glycogen synthase kinase-3 (GSK-3α/β), a protein-serine/threonine kinase whose activity is inhibited by PKB phosphorylation in response to growth factor stimulation. As a major downstream effector of PKB, evidence of GSK-3 phosphorylation was sought to provide additional confirmation that PI3-kinase was activated in this system.

As expected, MCP-1 stimulation of THP-1 cells resulted in a rapid and transient phosphorylation of PKB. Activation was evident 2 m after stimulation and had almost returned to basal levels after 10 m (Figure 4.12). This response was partially inhibited by pre-treatment with wortmannin (50nM).

THP-1 cells were shown to have constitutive activation of GSK-3 that was sensitive to wortmannin. MCP-1 induced a time-dependent increase in phosphorylated GSK-3 that was evident after 2 m, and still detectable 10 m post-stimulation - The induction of GSK-3 activity by MCP-1 was also sensitive to wortmannin. Although the kinetic profile was similar to that of PKB, the activation of GSK-3 appeared to be more sustained.

Figure 4.13 also demonstrates that MCP-2, -3 and -4 phosphorylates PKB and GSK-3. MCP-2 induced rapid activation of PKB that was detectable after 2 m and was still evident after 10 m (Figure 4.13a). All activity of PKB was abolished by pre-treatment with wortmannin. The phosphorylation of GSK-3 was slower and more transient. Whereby activation was detectable after 2 m, maximal at 5 minutes, and had almost returned to basal levels by 10 m. Wortmannin greatly reduced baseline activation of GSK-3, however, residual signalling was still evident in response to MCP-2.

Following stimulation of THP-1 cells with MCP-3, PKB activation and GSK-3 inactivation displayed similar kinetics (Figure 4.13B). Phosphorylation of both enzymes was rapid and displayed maximum activity after 2 m. Although reduced,

the activation of PKB and GSK-3 was sustained, and remained elevated 10 minutes post-stimulation. The inhibition of both PKB and GSK-3 by wortmannin further implied PI3K activation in this pathway. Stimulation of THP-1 cells with MCP-4 showed a modest increase in PKB and GSK-3 activity, both of which were sensitive to pre-treatment with wortmannin (Figure 4.13C). The kinetics were much slower than those observed with MCP-1, -2 and -3, as a clear response was not detectable until 10 m post-stimulation. In subsequent experiments, this delayed response was followed by a return to near basal levels after approximately 20-30 m (data not shown). In short, MCP-1, -2, -3 and -4, were all capable of eliciting phosphorylation of both PKB, and its downstream effector, GSK-3. Treatment with wortmannin attenuated these responses, and therefore provided further confirmation of the involvement of PI3-K as an upstream signalling component in this pathway.

Given that accumulation of MCP-1-induced accumulation PtdIns(3,4,5)P_3 in THP-1 cells appeared to be a G-protein-dependent event, the effects pertussis toxin treatment on CCR2-mediated PKB phosphorylation were investigated. Figure 4.14 shows that pertussis toxin completely inhibits MCP-1-induced PKB activation, but only partially inhibits MCP-2, -3, and -4-induced PKB activity. Even in the presence of this toxin there is evidence of residual signalling by MCP-2, -3, and –4.

Measurement of MCP-1-stimulated PI3-kinase activity In Vitro

From the HPLC analysis of total phospholipid production from THP-1 cells stimulated with CCR2 ligands, a role for more than one PI3K in the generation of PtdIns(3,4,5)P_3 was implied. It was important to clearly identify the PI3K isoforms involved in CCR2-mediated signalling. As previously discussed, another method used to identify PI3-kinase activation in this system was the assay of specific immunoprecipitated proteins (e.g. PI3K subunits, receptors, cellular proteins) for associated lipid kinase activity under *in vitro* conditions. Using distinct substrates such as PtdIns in the presence of [γ-^{32}P]-ATP (Morgan *et al*, 1990; Ward, 2000) the formation of radiolabelled PtdIns(3)P was detected using TLC and autoradiography.

Verification of expression of the prototypical p85/p110 heterodimer was achieved by western blotting of THP-1 cell lysates with anti-p85 antibody. Figure 4.15 shows the relative expression of p85 in THP-1 cells compared to Jurkat cells. *In vitro* lipid kinase assays demonstrated that stimulation of MCP-1 resulted in a time-dependent increase in p85 activity that was detectable at 30s and remained elevated for at least 15 minutes. The activity of immunoprecipitated p85 was sensitive to pre-treatment

with 10nM wortmannin, and PtdIns(3)P formation in response to MCP-1 ligation was reduced to basal levels.

Since PTX completely abrogated D-3-lipid accumulation, the activation of G protein - specific PI3K, PI3-Kγ, was assessed. Verification of the expression of this isoform was carried out by western blotting THP-1 cell lysates with an antibody towards p110γ (Fig 5.16A). Confirmation of p110γ activity was obtained by *in vitro* lipid kinase assay of MCP-stimulated THP-1 cells.

Figure 4.16B shows effects of various concentrations of MCP ligands on the generation of PtdIns(3)P from the immunoprecipitated p110γ activity. Although a reasonably high level of basal activity of this enzyme was observed, MCP-1 as well as MCP-2, -3 and –4 stimulated a concentration-dependent increase in this product (as assessed 30 seconds post-stimulation). Maximum responses were achieved with MCP-1, -2, -3 and –4 were 10nM, 100nM, 100nM and 10nM, respectively.

Further characterisation of immunoprecipitated p110γ activation was assessed in response to MCP-1-mediated activation. MCP-1-induced a time-dependent, biphasic increase in p110γ activity which peaked at 30s and 10 m. Figure 4.17 shows that treatment with wortmannin (10nM) inhibited p110γ activation to below basal levels. Similarly, 16- hour pre-treatment with pertussis toxin (100ng/ml) also inhibited all lipid kinase activity.

MCP-1-stimulated THP-1 cells revealed that PtdIns(3,4,5)P_3 accumulation was resistant to LY290042 and yet entirely sensitive to pertussis toxin. Given these findings, it was necessary to investigate the involvement the novel class II PI3-kinases, PI3K-C2α and PI3K-C2β, in this system. Indeed, MCP-1 stimulated an increase in the *in vitro* lipid kinase activity in both PI3K-C2α and PI3K-C2β imunoprecipitates (Figure 4.18A). The activity of both isoforms responded in a time-dependent manner, albeit with slightly different kinetics. PI3K-C2α appeared to be transiently activated with a detectable response observed 2 m post-stimulation, and by 10 m had declined back down to basal levels. Elevation of PI3K-C2β was also transient but slightly more sustained. Increases were detectable after 15 s, maximal at 5 m and returned to baseline activity at 10 m. Further characterisation of MCP-1-stimulated PI3K-C2β was performed in the presence of pertussis toxin and wortmannin (Figure 4.18B). Although reported to have limited sensitivity to PI3-kinase inhibitors, (Domin and Waterfield, 1997), pre-treatment with wortmannin as

well as pertussis toxin almost completely abrogated the MCP-1-induced *in vitro* lipid kinase activity of PI3K-C2β immunoprecipitates.

The lipid data demonstrated that MCP-1 stimulates a rapid and transient increase in PtdIns(3,4,5)P_3 compared with the slower, sustained increase in PtdIns(3,4)P_2. This could be explained by the concomitant activation of a PtdIns(3,4,5)P_3 5-phosphatase, which has been identified in other systems. To address this possibility, the THP-1 cell line was screened for the expression of SH2-domain-containing inositol polyphosphate 5-phosphatase (SHIP) (Figure 4.19). Immunoblotting THP-1 cell lysates with anti-human SHIP antibody revealed a protein that corresponded to 145kDa, consistent with expression of SHIP. The A20 cell line and peripheral blood mononuclear cells (PBMCs) were positive controls for this protein. The Jurkat T cell line, provided a suitable negative control as these cells characteristically lack expression of the phosphatases SHIP and PTEN (S. Burgess, unpublished data).

Immunoblotting anti-SHIP immunoprecipitates derived from resting and stimulated THP-1 cells with antiphosphotyrosine mAb revealed that 145kDa protein (SHIP) was modestly tyrosine phosphorylated following MCP-1 stimulation. Although there was basal activity of this protein, there was a concentration-dependent response with maximum phosphorylation observed using 10nM MCP-1 (Figure 4.20). A preliminary experiment also showed that SHIP phosphorylation was time-dependent (n=1). A detectable response was observed 30 s post-stimulation and remained elevated after 10 m.

The Role of PI3-kinase on CCR2-mediated chemotaxis

The involvement of PI3K(s) on CCR2-mediated chemotaxis was characterised with the use of phamacological PI3-kinase inhibitor, wortmannin (Figure 4.21 A - D). As discussed in Section 4, MCP-1, as well as MCP-2, -3 and -4, induce chemotaxis in THP-1 cells in a concentration-dependent manner that is characteristic of chemokine-dependent chemotaxis. THP-1 cell chemotaxis towards MCP-1 at a concentration known to elicit optimal migration was almost completely inhibited by pre-treatment of cells with wortmannin (IC_{50} = 25nM). Interestingly, residual migration towards MCP-1 was observed even at the upper limit of wortmannin concentration (100nM) which implies that there is only a partial dependency on PI3-K for MCP-1-induced migration. Chemotaxis towards MCP-2, -3 and -4 was also sensitive to PI3K

inhibition, albeit to much lower concentrations of wortmannin (IC_{50} = 2nM, <0.1nM, <0.1nM, respectively).

Table 4.1. Summary of the effects of CCR2 ligands on D-3-phosphorylated lipid generation and downstream effectors of PI3-kinase in THP-1 cells.

	Increase in PtdIns(3,4,5)P_3 ?		**Increase in PtdIns(3,4)P_2 ?**	
MCP-1	✓	Insensitive to LY. Sensitive to PTX.	✓	Insensitive to LY
MCP-2	✓	Insensitive to LY	✓	Insensitive to LY
MCP-3	✓	Insensitive to LY	✓	Insensitive to LY
MCP-4	✓	Insensitive to LY	✓	Insensitive to LY
	Activation of PKB?		**Activation of GSK-3?**	
MCP-1	✓	Partial sensitivity to WM. Sensitive to PTX	✓	Partial sensitivity to WM
MCP-2	✓	Sensitive to WM Partial sensitivity to PTX	✓	Partial sensitivity to WM
MCP-3	✓	Sensitive to WM Partial sensitivity to PTX	✓	Sensitive to WM
MCP-4	✓	Sensitive to WM Partial sensitivity to PTX	✓	Sensitive to WM

Table 4.2 PI3-kinase Isoforms activated by ligands of CCR2

	p85 ?		**p110γ ?**		**PI3K-C2α/β ?**	
MCP-1	✓	sensitive to WM. sensitive to PTX	✓	optimal conc. = 10nM sensitive to WM. sensitive to PTX	✓	PI3-KC2α
					✓	PI3-KC2β Sensitive to WM Sensitive to PTX
MCP-2	-	-	✓	optimal conc. = 100nM	-	-
MCP-3	-	-	✓	optimal conc. = 100nM	-	-
MCP-4	-	-	✓	optimal conc. = 10nM	-	-

Legend: ✓; activation, - ;response not assessed, LY, LY290042, WM; wortmannin, PTX; pertussis toxin

Figure 4.1 Typical HPLC trace for the separation and analysis of phosphatidyl-inositol(3,4,5)P_3 generation in Jurkat cells

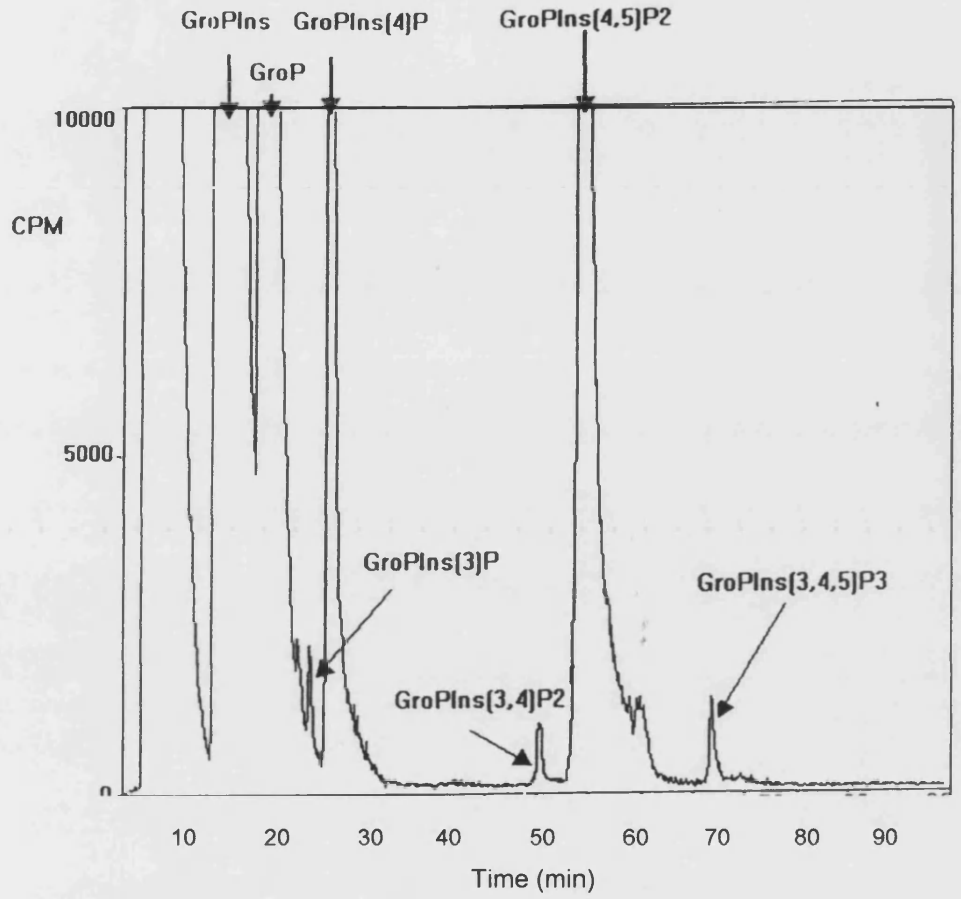
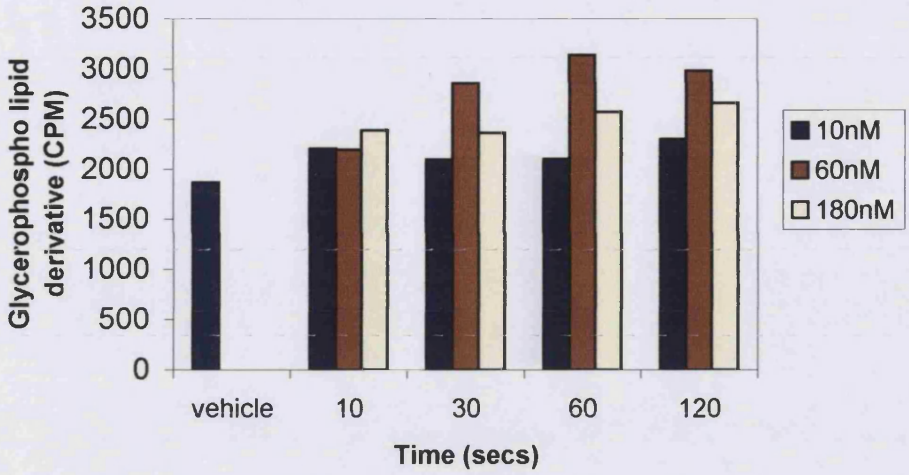


Figure 4.1 Typical HPLC trace for the separation and analysis of phosphatidyl-inositol(3,4,5)P_3 generation in Jurkat cells. Jurkat cells ($1x10^7$ cell/point) were metabolically labelled with [^{32}P], stimulated with 10μg/ml UCHT1 for 1 minute, and phospholipids were extracted as described in Materials and Methods. The resulting glycerophosphoryl sample derivatives (GroPtdIns lipids) were separated by anion exchange HPLC and analysis conducted by the Flo-One radiomatic program. Retention times of eluate peaks were verified for each experiment against ^3H-labelled phosphoinositide lipid standards. Relevant phospholipid peaks are indicated and expressed in CPM/Time (min). Trace is taken from one experiment, and is representative of all lipid extraction experiments.

Figure 4.2 Effect of MCP-1-stimulation on PtdIns(3,4,5)P_3 and PtdIns(3,4)P_2 generation in THP-1 cells

A) Accumulation of glyceroderivative, PtdIns(3,4,5)P_3



B) Accumulation of glyceroderivative, PtdIns(3,4)P_2

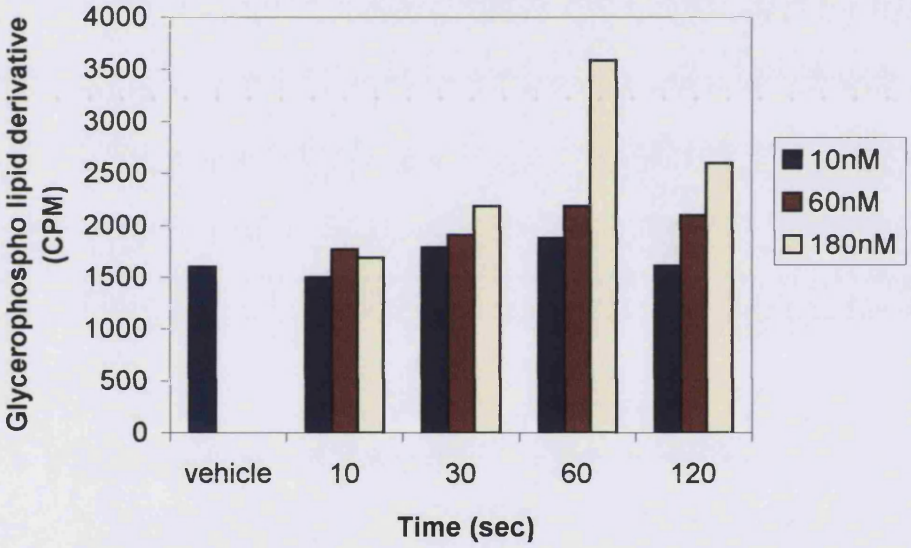
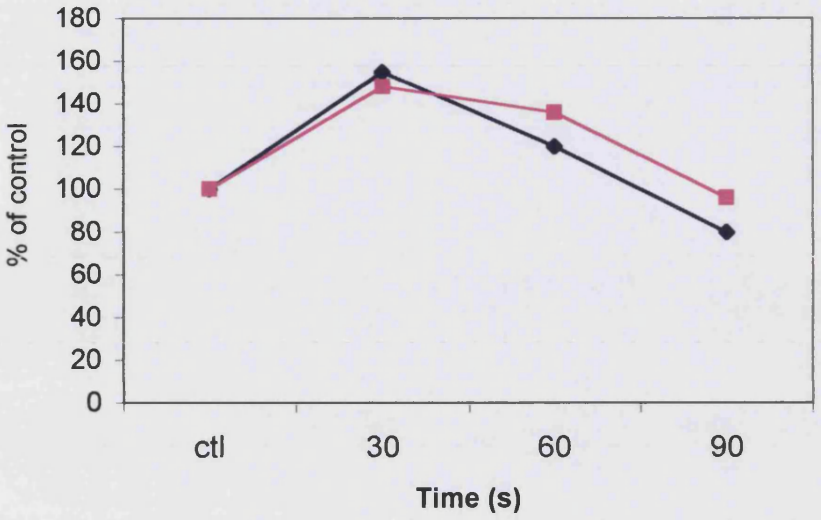


Figure 4.2. Effect of MCP-1 stimulation on PtdIns(3,4)P_2 and PtdIns(3,4,5)P_3 generation in THP-1 cells. 1 x 10^7 cells/point were metabolically labelled with ^{32}P. Cells were stimulated with vehicle (0.05% BSA) or various concentrations of MCP-1 for the indicated times. Following stimulation, phospholipids were extracted, deacylated and the glycerophosphorylinositol derivatives of PtdIns(3,4,5)P_3 ***(A)*** and PtdIns(3,4)P_2 ***(B)*** were analysed using HPLC as described under 'Materials and Methods'. Data are from a single experiment and are representative of at least three separate experiments.

Figure 4.3 Effect of LY290042 on MCP-1-induced generation of PtdIns(3,4)P_2 and PtdIns(3,4,5)P_3 in THP-1 cells.

A) Accumulation of glyceroderivative, PtdIns(3,4,5)P_3



B) Accumulation of glyceroderivative, PtdIns(3,4)P_2

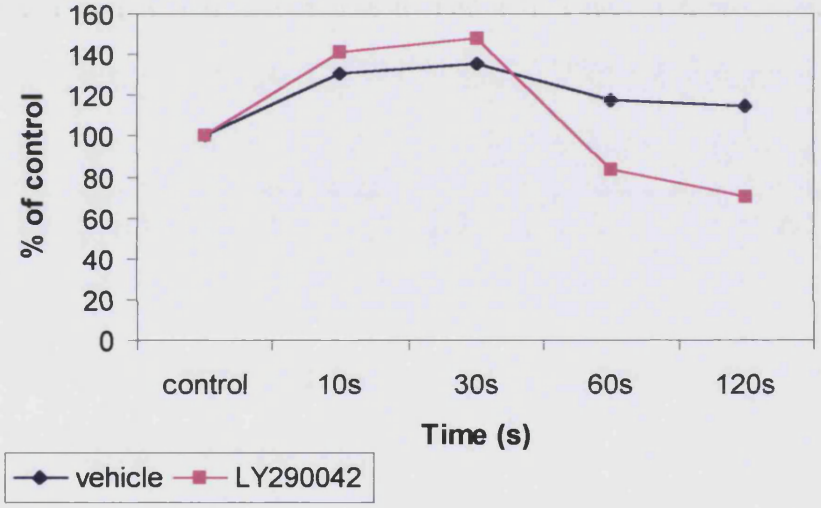


Figure 4.3. Effect of LY290042 on MCP-1-induced accumulation of PtdIns(3,4)P_2 and PtdIns(3,4,5)P_3 in THP-1 cells. 1 x 10^7 cells/point were metabolically labelled with ^{32}P and pre-treated for 10 minutes with either vehicle (0.01% DMSO) or 10μM LY-290042. Cells were then stimulated with vehicle (0.05% BSA) or MCP-1 (60nM) for various times. Following stimulation, phospholipids were extracted, deacylated and the glycerophosphorylinositol derivatives of PtdIns(3,4,5)P_3 ***(A)*** and PtdIns(3,4)P_2 ***(B)*** were analysed using HPLC as described under 'Materials and Methods'. Data are from a single experiment and are representative of at least three separate experiments.

Figure 4.4. The Effect of Pertussis toxin on MCP-1-induced PtdIns(3,4,5)P_3 accumulation in THP-1 cells

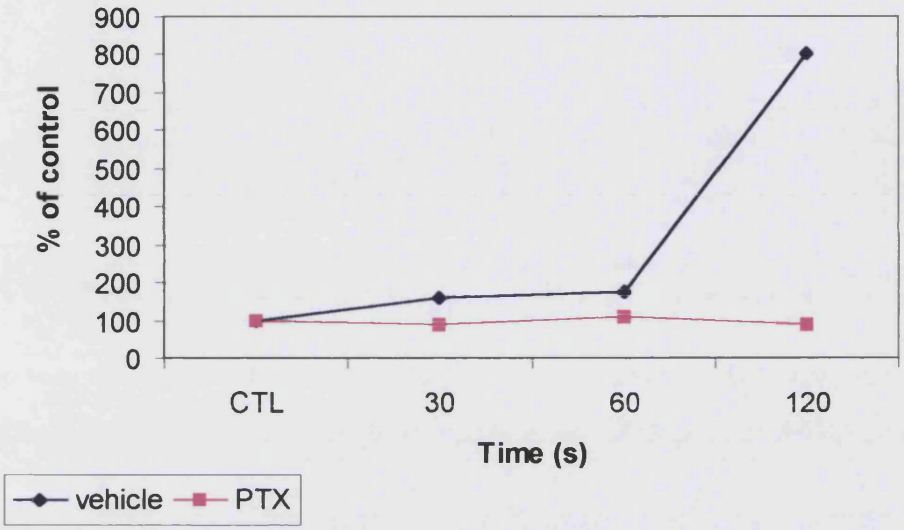
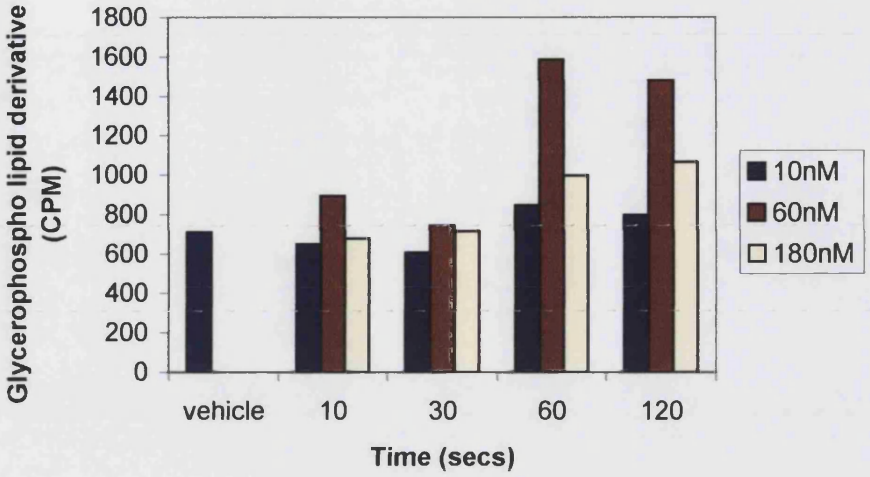


Figure 4.4. Effect of Pertussis toxin on MCP-1-stimulated generation of PtdIns(3,4,5)P_3 and PtdIns(3,4,)P_2 in THP-1 cells. Following pre-treatment for 16 hours with vehicle (0.01% dH_2O) or 100ng/ml pertussis toxin, $1x10^7$ cells/point were metabolically labelled with ^{32}P. Cells were then stimulated with vehicle (0.05% BSA) or MCP-1 (60nM) for the indicated times. Following stimulation, phospholipids were extracted, deacylated and the glycerophosphorylinositol derivative of PtdIns(3,4,5)P_3 was analysed using HPLC as described under 'Materials and Methods'. Data are from a single experiment and are representative of two separate experiments.

Figure 4.5. Effect of MCP-2-stimulation on PtdIns(3,4,5)P_3 and PtdIns(3,4)P_2 generation in THP-1 cells

A) Accumulation of glyceroderivative, PtdIns(3,4,5)P_3



) Accumulation of lyceroderivative, tdIns(3,4)P_2

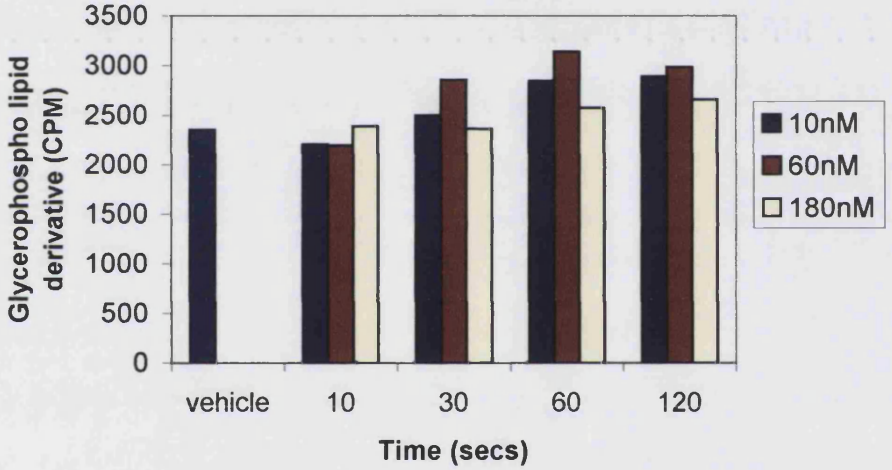
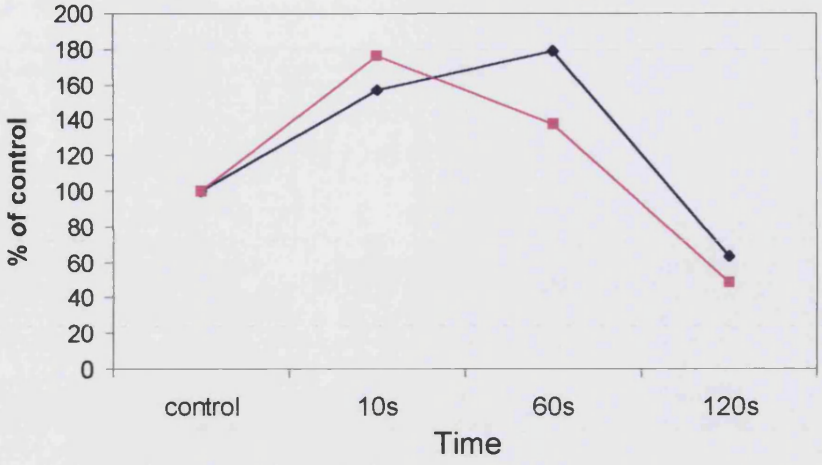


Figure 4.5. Effect of MCP-2 stimulation on PtdIns(3,4)P_2 and PtdIns(3,4,5)P_3 generation in THP-1 cells. 1 x 10^7 cells/point were metabolically labelled with ^{32}P. Cells were stimulated with vehicle (0.05% BSA) or various concentrations of MCP-2 for the indicated times. Following stimulation, phospholipids were extracted, deacylated and the glycerophosphorylinositol derivatives of PtdIns(3,4,5)P_3 ***(A)*** and PtdIns(3,4)P_2 ***(B)*** were analysed using HPLC as described under 'Materials and Methods'. Data are from a single experiment and are representative of at least three separate experiments.

Figure 4.6. Effect of LY290042 on MCP-2-induced generation of PtdIns(3,4)P_2 and PtdIns(3,4,5)P_3 in THP-1 cells.

A) Accumulation of glyceroderivative, PtdIns(3,4,5)P_3



B) Accumulation of glyceroderivative, PtdIns(3,4)P_2

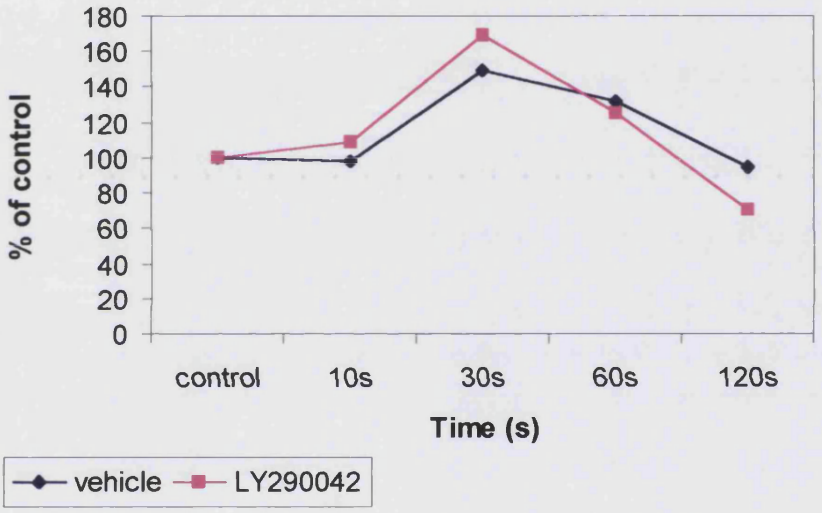
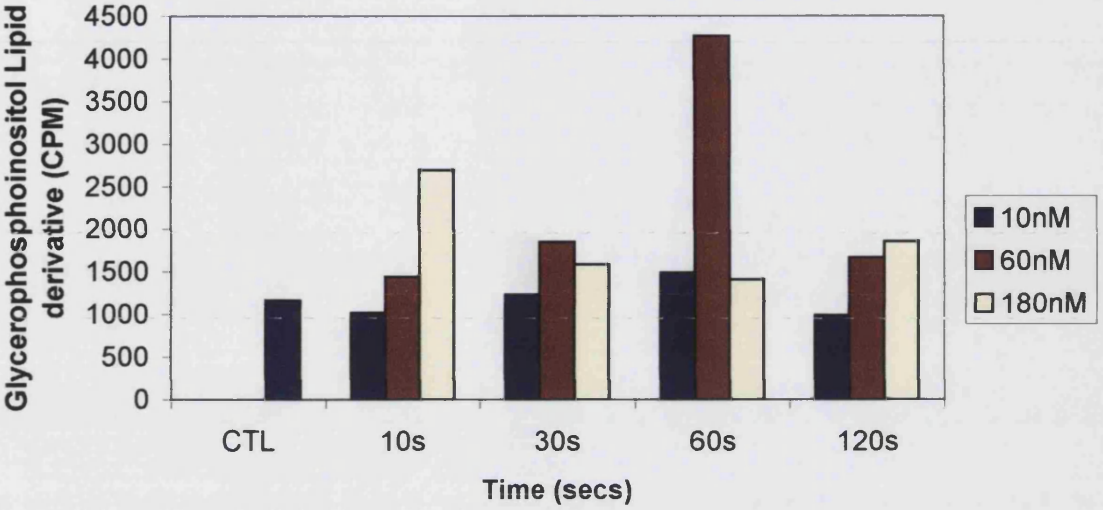


Figure 4.6. Effect of LY290042 on MCP-2-induced generation of PtdIns(3,4)P_2 and PtdIns(3,4, 5)P_3 in THP-1 cells. 1 x 10^7 cells/point were metabolically labelled with ^{32}P and pre-treated for 10 minutes with either vehicle (0.01% DMSO) or 10μM LY-290042. Cells were then stimulated with vehicle (0.05% BSA) or MCP-2 (60nM). Following stimulation, phospholipids were extracted, deacylated and the glycerophosphorylinositol derivatives of PtdIns(3,4,5)P_3 ***(A)*** and PtdIns(3,4)P_2 ***(B)*** were analysed using HPLC as described under 'Materials and Methods'. Data are from a single experiment and are representative of at least three separate experiments.

Figure 4.7. Effect of MCP-3-stimulation on PtdIns(3,4,5)P_3 and PtdIns(3,4)P_2 generation in THP-1 cells

A) Accumulation of glyceroderivative, PtdIns(3,4,5)P_3



B) Accumulation of glyceroderivative, PtdIns(3,4)P_2

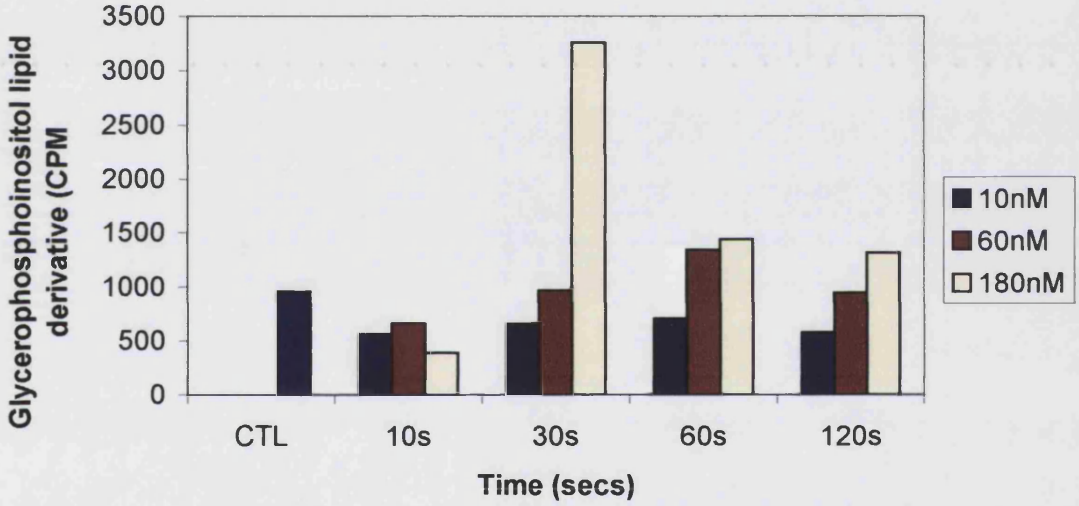
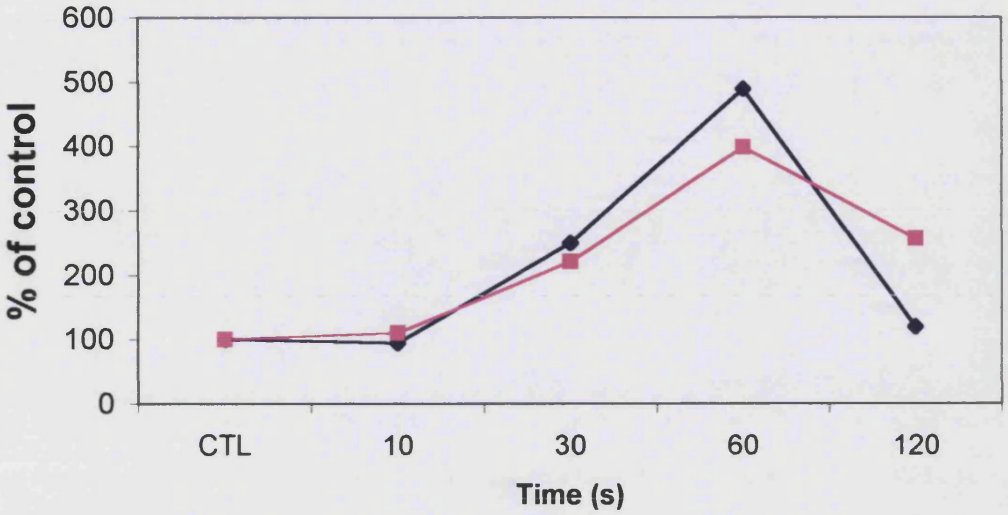


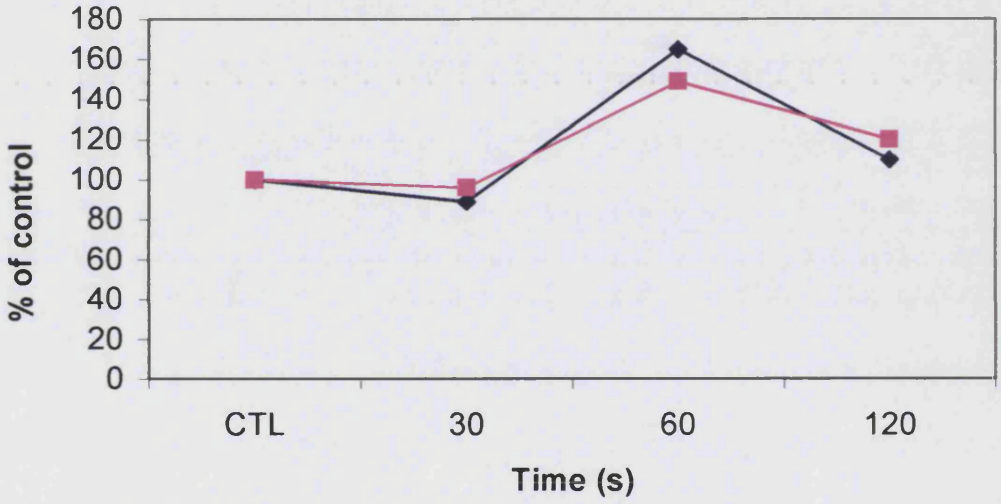
Figure 4.7. Effect of MCP-3 stimulation on PtdIns(3,4)P_2 and PtdIns(3,4,5)P_3 generation in THP-1 cells. 1 x 10^7 cells/point were metabolically labelled with ^{32}P, and then stimulated with vehicle (0.05% BSA) or various concentrations of MCP-3. Following stimulation, phospholipids were extracted, deacylated and the glycerophosphorylinositol derivatives of PtdIns(3,4,5)P_3 ***(A)*** and PtdIns(3,4)P_2 ***(B)*** were analysed using HPLC as described under 'Materials and Methods'. Data are from a single experiment and are representative of at least three separate experiments.

Figure 4.8. Effect of LY-290042 on MCP-3-induced generation of PtdIns(3,4)P_2 and PtdIns(3,4,5)P_3 in THP-1 cells.

A) Accumulation of glyceroderivative, PtdIns(3,4,5)P_3



B) Accumulation of glyceroderivative, PtdIns(3,4)P_2



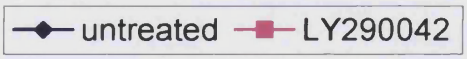
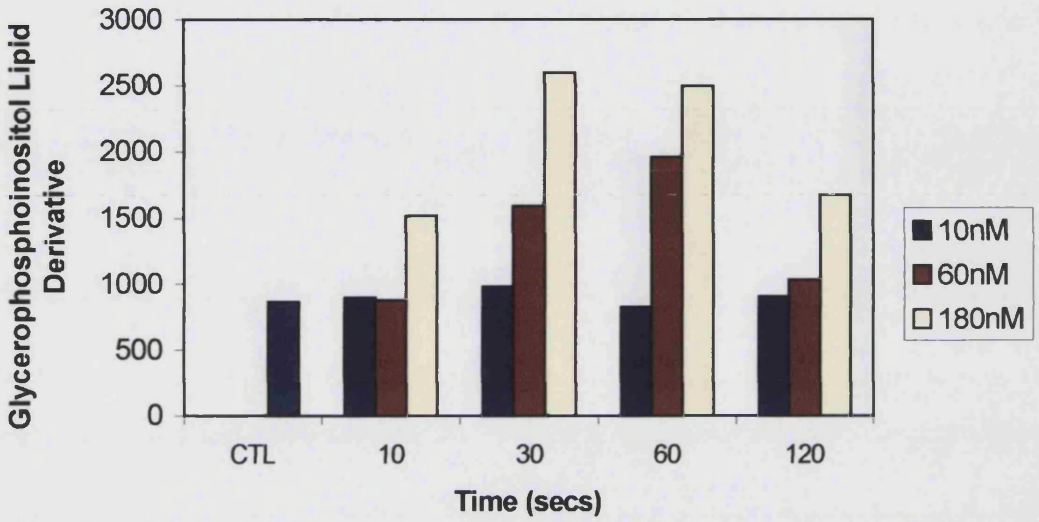


Figure 4.8. Effect of MCP-3-induced generation of PtdIns(3,4)P_2 and PtdIns(3,4,5)P_3 in THP-1 cells. 1 x 10^7 cells/point were metabolically labelled with ^{32}P and pre-treated for 10 minutes with either vehicle (0.01% DMSO) or 10μM LY-290042. Cells were then stimulated with vehicle (0.05% BSA) or MCP-3 (60nM). Following stimulation, phospholipids were extracted, deacylated and the glycerophosphorylinositol derivatives of PtdIns(3,4,5)P_3 ***(A)*** and PtdIns(3,4)P_2 ***(B)*** were analysed using HPLC as described under 'Materials and Methods'. Data are from a single experiment and are representative of at least three separate experiments.

Figure 4.9 Effect of MCP-4-stimulation on PtdIns(3,4,5)P_3 and PtdIns(3,4)P_2 generation in THP-1 cells

A) Accumulation of glyceroderivative, PtdIns(3,4,5)P_3



B) Accumulation of glyceroderivative, PtdIns(3,4)P_2

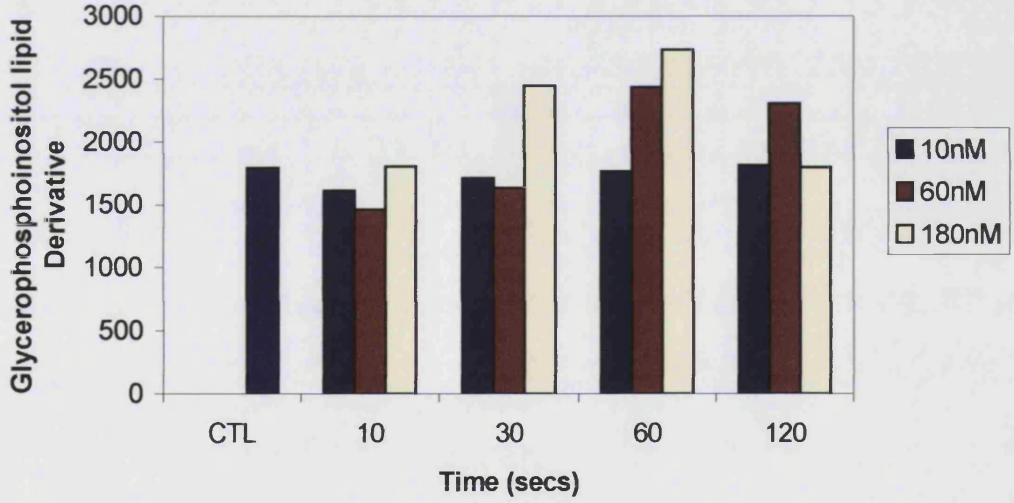
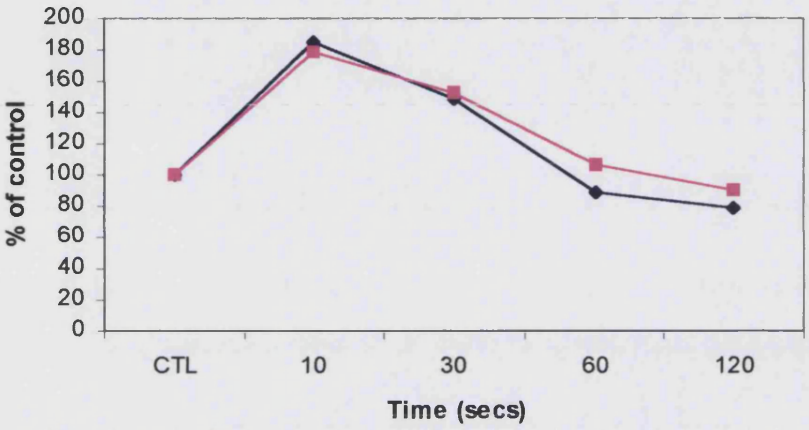


Figure 4.9. Effect of MCP-4 stimulation on PtdIns(3,4)P_2 and PtdIns(3,4,5)P_3 generation in THP-1 cells. 1 x 10^7 cells were metabolically labelled with ^{32}P and then stimulated with vehicle (0.05% BSA) or various concentrations of MCP-4. Following stimulation, phospholipids were extracted, deacylated and the glycerophosphorylinositol derivatives of PtdIns(3,4,5)P_3 ***(A)*** and PtdIns(3,4)P_2 ***(B)*** were analysed using HPLC as described under 'Materials and Methods'. Data are from a single experiment and are representative of at least three separate experiments.

Figure 4.10. Effect of LY290042 on MCP-4-induced accumulation of PtdIns(3,4)P_2 and PtdIns(3,4,5)P_3 in THP-1 cells.

A) Accumulation of glyceroderivative, PtdIns(3,4,5)P_3



B) Accumulation of glyceroderivative, PtdIns(3,4)P_2

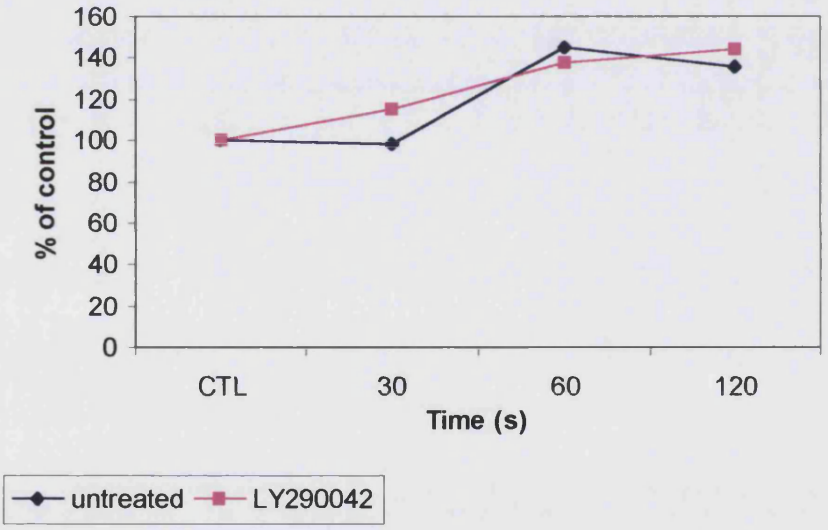


Figure 4.10. Effect of LY290042 on MCP-4-induced accumulation of PtdIns(3,4)P_2 and PtdIns(3,4, 5)P_3 in THP-1 cells. 1 x 10^7 cells/point were metabolically labelled with ^{32}P and pre-treated for 10 minutes with either vehicle (0.01% DMSO) or 10μM LY-290042. Cells were then stimulated with vehicle (0.05% BSA) or MCP-4 (60nM). Following stimulation, phospholipids were extracted, deacylated and the glycerophosphorylinositol derivatives of PtdIns(3,4,5)P_3 ***(A)*** and PtdIns(3,4)P_2 ***(B)*** were analysed using HPLC as described under "Materials and Methods". Data are from a single experiment and are representative of at least three separate experiments.

Figure 4.11. Comparison of MCP-1, -2, -3 and -4-induced generation of PtdIns(3,4,5)P_3 in THP-1 cells

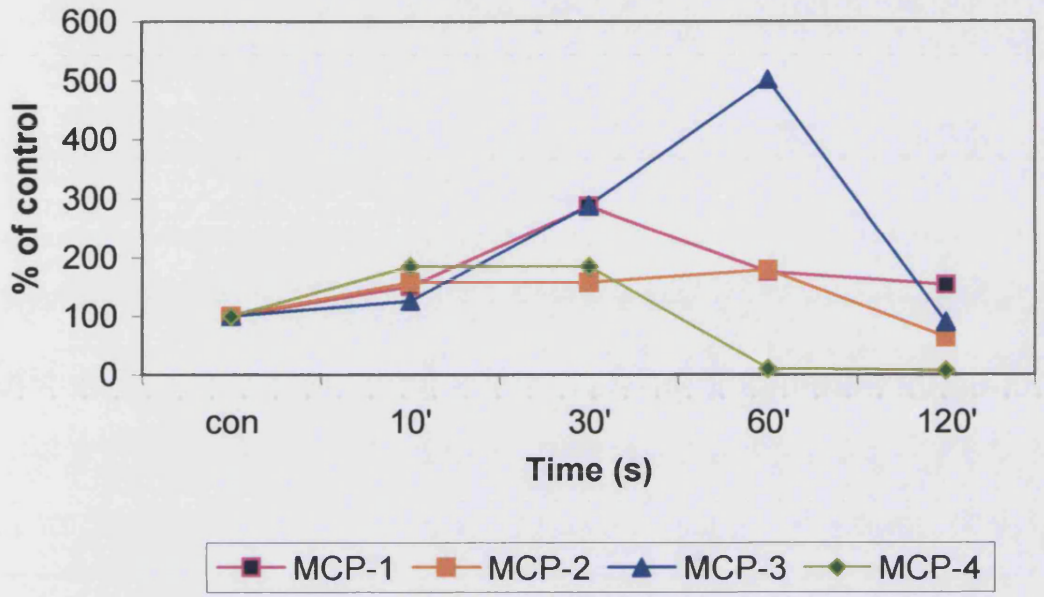


Figure 4.11. Effect of MCP-1, -2, -3 and -4 stimulation on PtdIns(3,4,5)P_3 generation in THP-1 cells. 1 x 10^7 cells were metabolically labelled with ^{32}P and then stimulated with vehicle (0.05% BSA) or MCP-1, -2, -3 (60nM) and MCP-4 (180nM). Following stimulation, phospholipids were extracted, deacylated and the glycerophosphorylinositol derivative of PtdIns(3,4,5)P_3 were analysed using HPLC as described under 'Materials and Methods'. Data are from a single experiment and are representative of at least two separate experiments.

Figure 4.12/13 Effect of wortmannin on MCP-1, MCP-2, -3 and -4-induced activation of PKB in THP-1 cells.

Fig 4.12

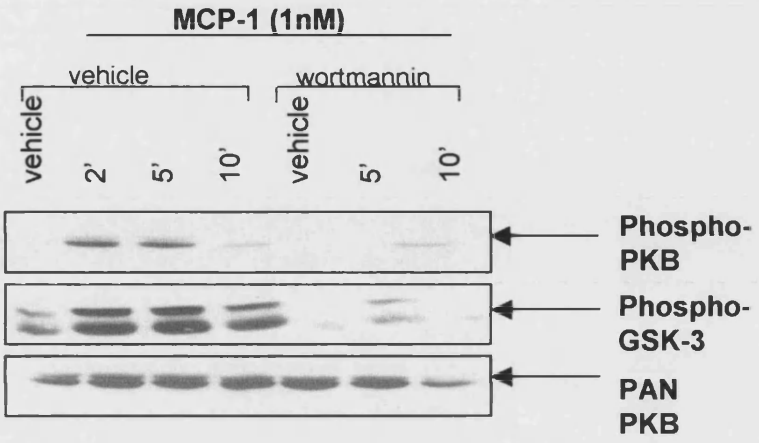
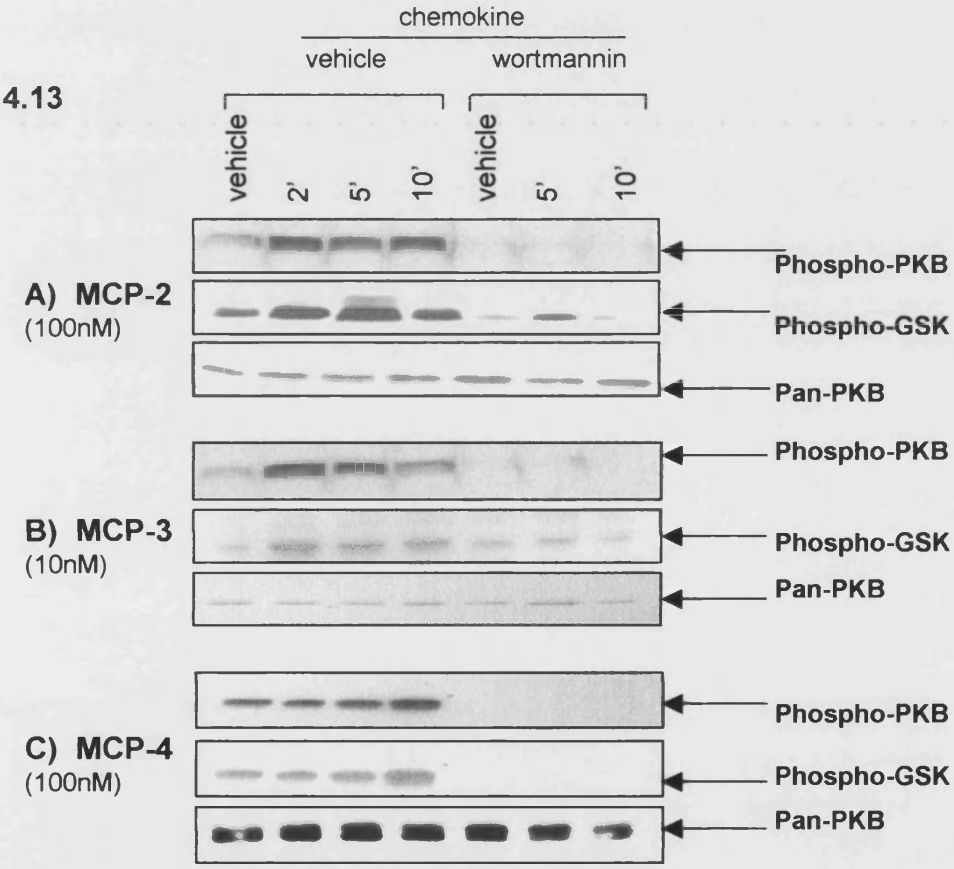


Fig 4.13



Figures 4.12 and 4.13. Effect of wortmannin on MCP-2, -3 and -4-induced activation of PKB in THP-1 cells. THP-1 cells ($1x10^6$ cells per point) were pre-treated for 15 minutes with vehicle (0.01% DMSO) or 50nM wortmannin. Cells were then stimulated with vehicle (0.05% BSA) MCP-1 **(Fig 4.12)** MCP-2 **(Fig 4.13A),** MCP-3 **(Fig 4.13B)** or MCP-4 **(Fig 4.13C)** for the times indicated. For each time course, whole cell lysates were split into four equal volumes. All samples were resolved separately by SDS-PAGE, and electrophoretically transferred to a nitrocellulose membrane. Lysates were immunoblotted with either anti-phospho-PKB or phospho-GSK-3. The remaining lysates were immunoblotted with anti- PKB to verify equal loading of proteins. The results are representative of at least three experiments.

Figure 4.14. The Effect of Pertussis toxin on PKB activation in THP-1 cells

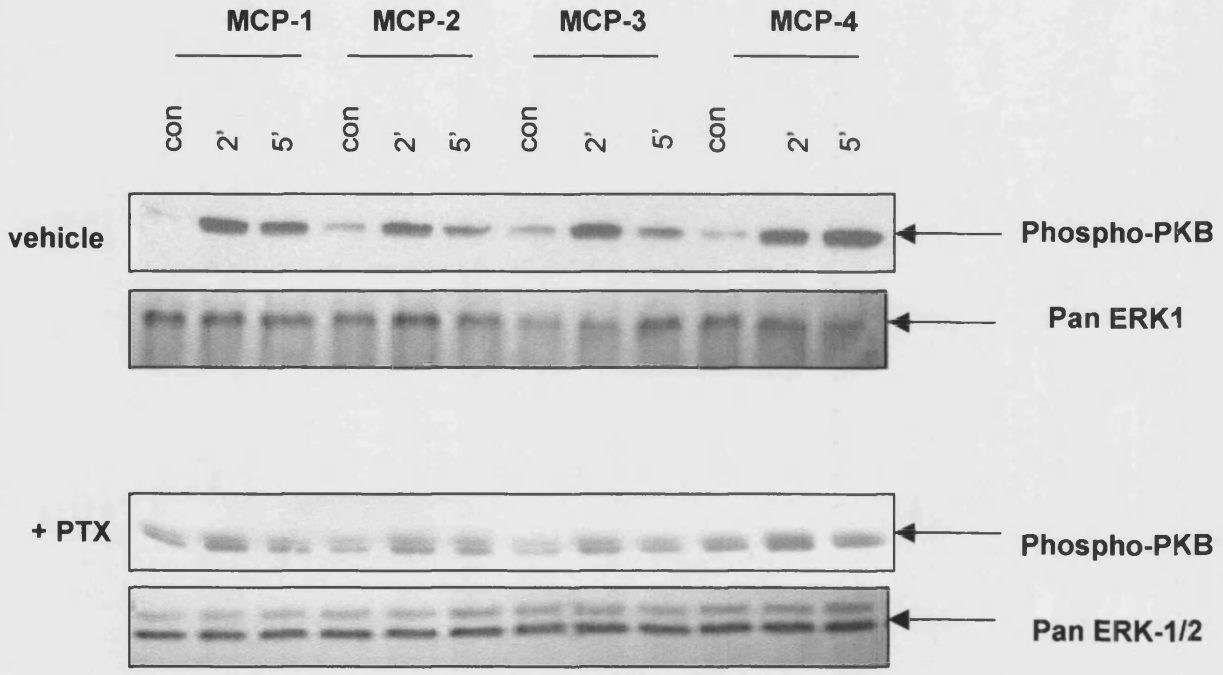


Figure 4.14. The Effect of pertussis toxin on MCP-1, -2, -3, and -4-induced PKB activation in THP-1 cells. THP-1 cells ($1x10^6$ per point) were pre-treated at 37°C for 16 hours with either vehicle (0.01% DMSO) or 100ng/ml PTX. Cells were then stimulated with vehicle (0.05% BSA) or MCP-1, -2, -3, -4 for the times indicated. Total cell lysates (2.5×10^5 cell equivalents per lane) were resolved separately by SDS-PAGE, electrophoretically transferred to a nitrocellulose membrane and immunoblotted with phospho-PKB or pan ERK/ERK-1/2 antibodies (to ensure equal loading of proteins). The results are representative of two experiments.

Figure 4.15 Verification of expression and activity of Class I_A PI3K isoform, p85, in THP-1 cells

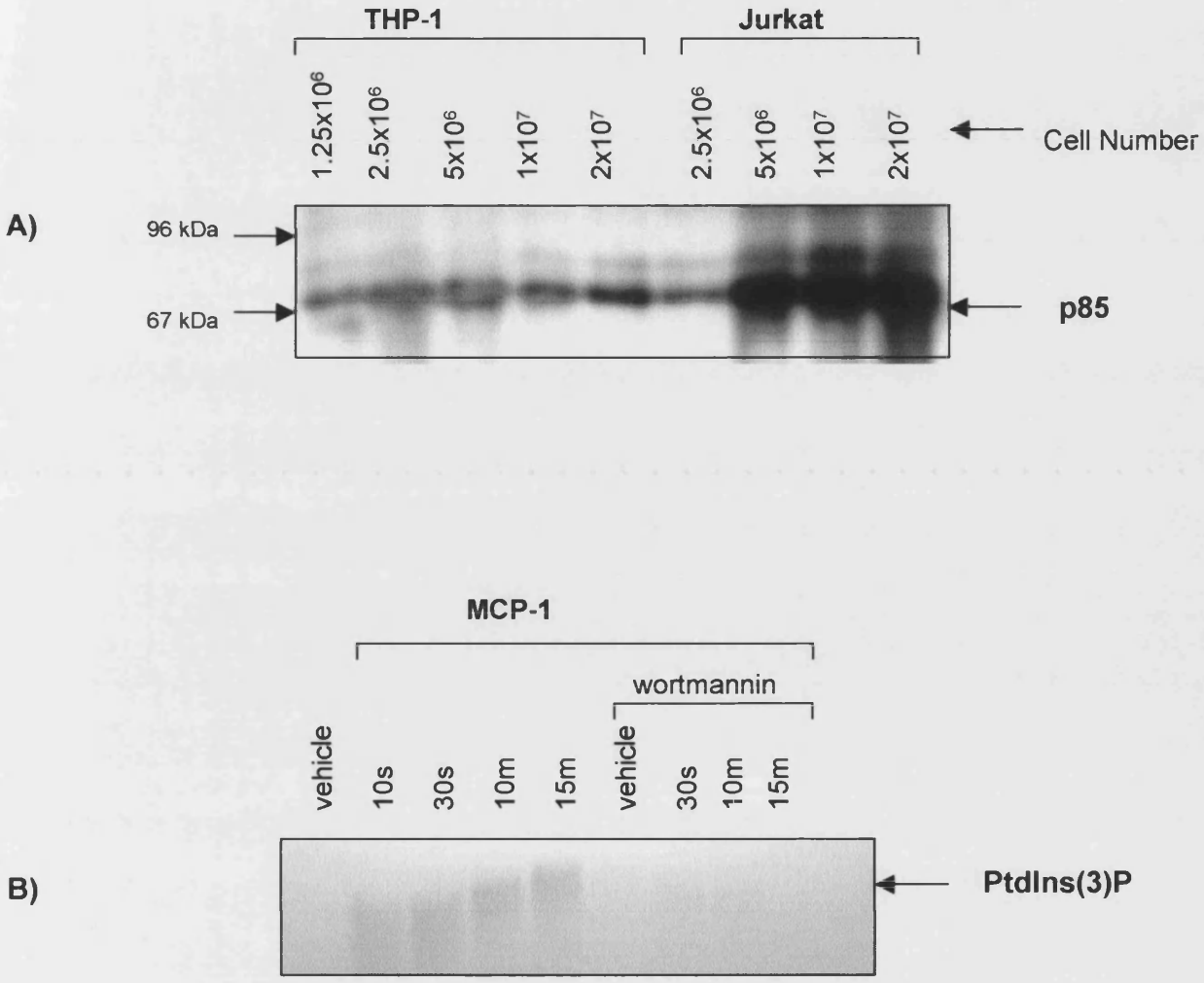


Figure 4.15A. Expression of p85 in THP-1 cells. Resting THP-1 cells from $1.25x10^6$ - $2x10^7$ cell equivalents were lysed, resolved separately by SDS-PAGE, and electrophoretically transferred to a nitrocellulose membrane as described in 'Materials and Methods'. Lysates were immunoblotted with anti-p85 mAb. Jurkat cell lysates were used as a positive control. Results are from one experiment and representative of another 3 independent experiments.

Figure 4.15B. MCP-1 stimulates increases in p85 immunoprecipitates in THP-1 cells. $1x10^7$ THP-1 cells were stimulated for various times with 10nM MCP-1. Cells were lysed, and lysates were subjected to immunoprecipitation with an anti-p85 mAb. The washed immunoprecipiates were analysed for PI3K activity using PI as a substrate. Extraction and TLC separation of the lipid products were performed as described under 'Materials and Methods'. Lipids were detected by exposure to film at -70°C. Results are representative of three separate experiments.

Figure 4.16 Verification of expression and activity of Class I_B PI3K isoform, p110γ, in THP-1 cells

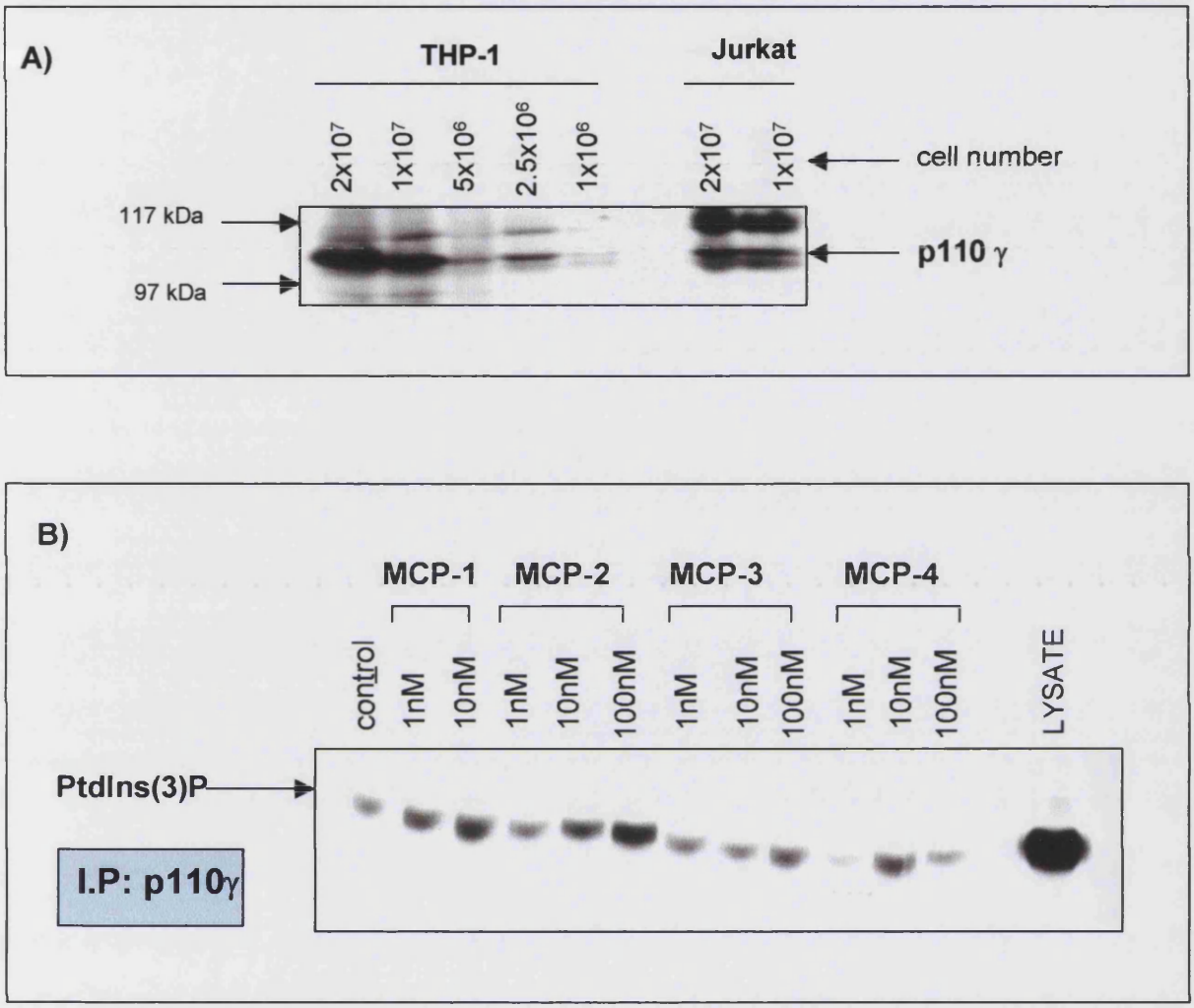


Figure 4.16A Expression of p110γ in THP-1 cells. Resting THP-1 cells from $1x10^6$ - $2x10^7$ cell equivalents were lysed, resolved separately by SDS-PAGE, and electrophoretically transferred to a nitrocellulose membrane as described in 'Materials and Methods'. Lysates were immunoblotted with anti-p110γ mAb. Jurkat cell lysates were used as a positive control. Results are from one experiment and representative of another 2 independent experiments.

Figure 4.16B. MCP-1 stimulates increases in p110γ immunoprecipitates in THP-1 cells. $1x10^7$ THP-1 cells were stimulated for 1 minute with various concentrations of MCP-1, -2, -3 and -4. Cells were lysed, and lysates were subjected to immunoprecipitation with an anti-p110γ mAb. The washed immunoprecipiates were analysed for PI3K activity using PI as a substrate. Extraction and TLC separation of the lipid products were performed as described under 'Materials and Methods'. Lipids were detected by exposure to film at -70°C. Results are representative of four separate experiments.

Figure 4.17. Effects of wortmannin and pertussis toxin on *in vitro* PI 3-kinase activity.

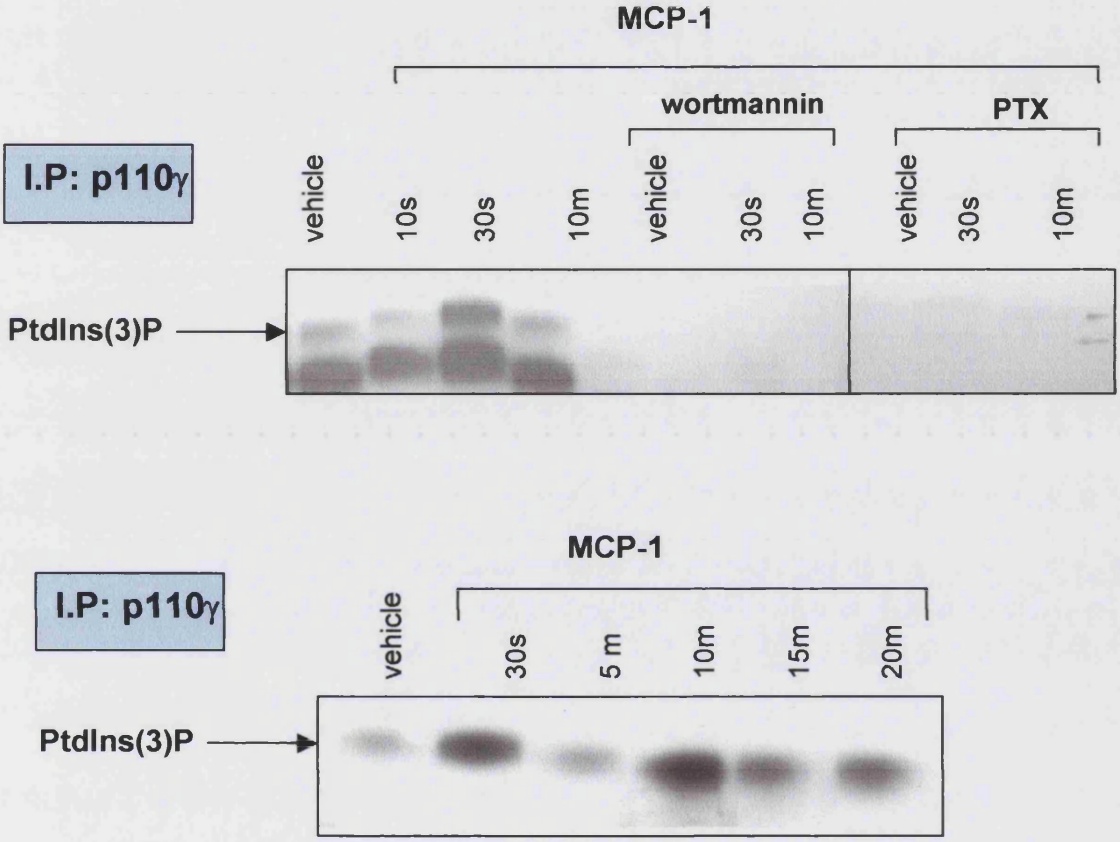


Figure 4.17. Effects of wortmannin and pertussis toxin on *in vitro* PI 3-kinase activity. **A)** THP-1 cells were pre-treated for 16 h with 100ng/ml pertussis toxin, 15 minutes with 50nM wortmannin or vehicle (0.01% DMSO) and then $1x10^7$ cells/point were stimulated with MCP-1(10nM) for the indicated times. Cells were lysed, and lysates were subjected to immunoprecipitation with an anti-p110γ mAb. The washed immunoprecipiates were analysed for PI3K activity using PI as a substrate. **B)** Extended time-course of MCP-1-stimulated p110γ immunoprecipitates in the absence of inhibitors. Extraction and TLC separation of the lipid products were performed as described under 'Materials and Methods'. Lipids were detected by exposure to film at -70°C. Results are representative of four separate experiments.

Figure 4.18a *In vitro* lipid kinase assay of MCP-1 stimulated THP-1 cells: Analysis of the Class II PI3K-C2 isoforms

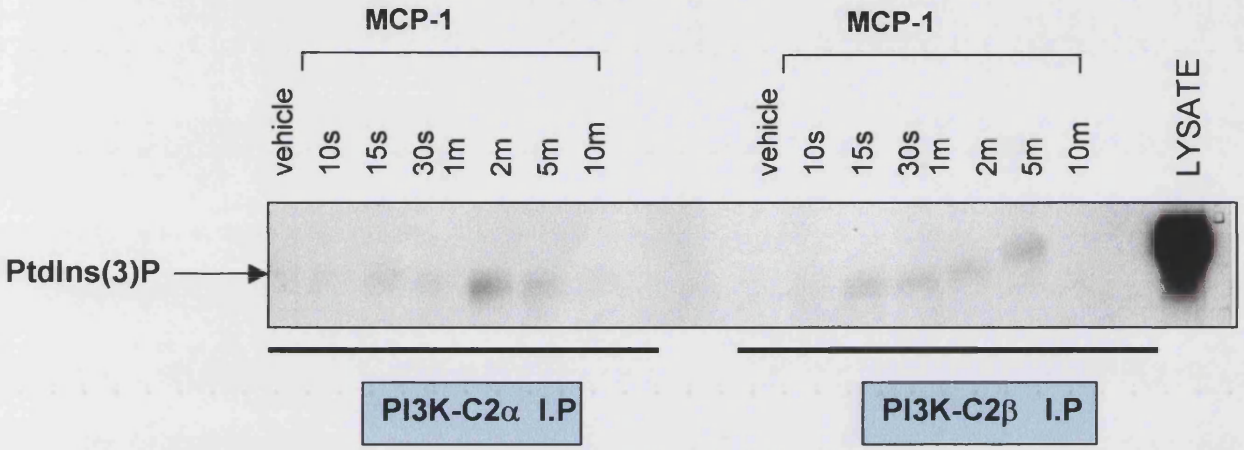


Figure 4.18a. *In vitro* lipid kinase assay of MCP-1 stimulated THP-1 cells: Analysis of the Class II PI3K-C2 isoforms. $1x10^7$ cells/point were stimulated at 37°C with 10nM MCP-1 for various times. Cells were lysed, and lysates subjected to immunoprecipitation with anti-PI3K-C2α and PI3K-C2β polyclonal antibodies. The washed immunoprecipitates were analysed for PI kinase activity using PI as a substrate. Extraction and TLC separation of the lipid products were performed as described under 'Materials and Methods'. Lipids were detected by exposure to film at -70°C. The data is representative of three separate experiments.

Figure 4.18b. *In vitro* lipid kinase assay of MCP-1 stimulated THP-1 cells: Analysis of the Class II PI3K-C2 isoforms.

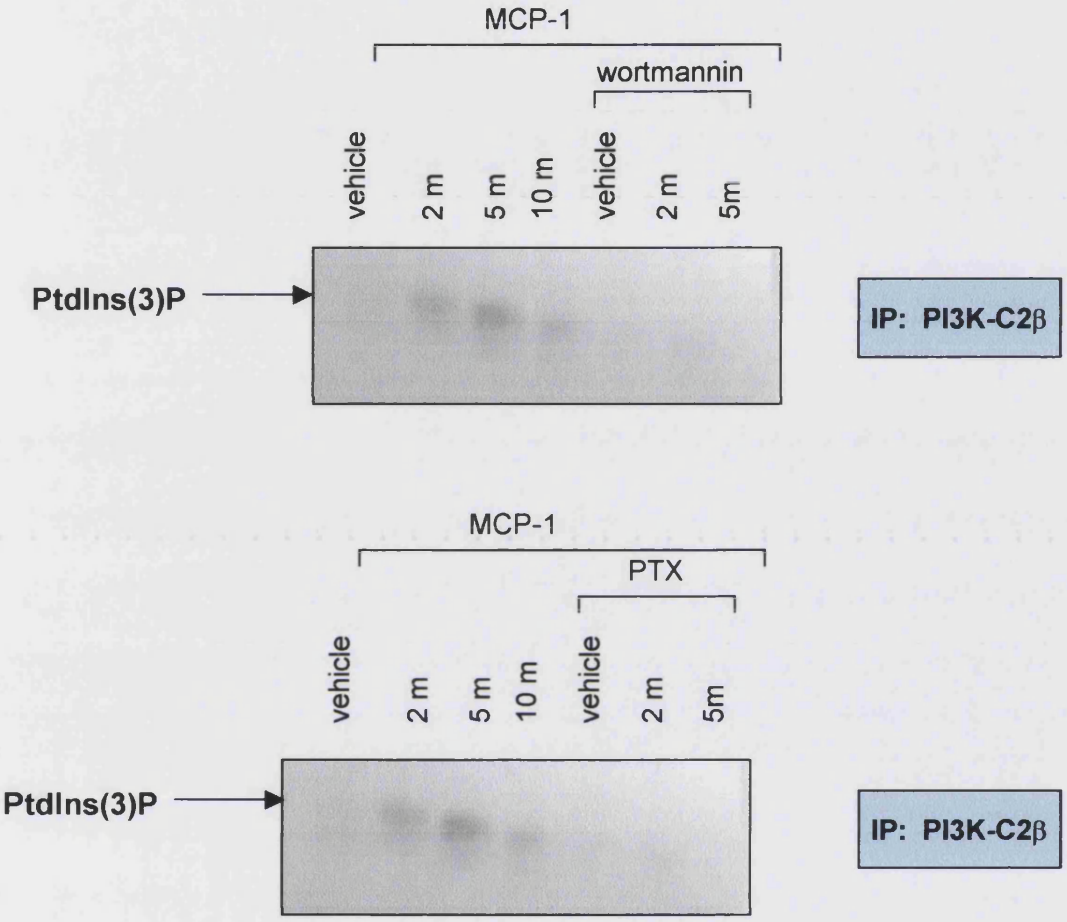


Figure 4.18b. MCP-1-stimulates increases in PI3K-C2β immunoprecipitates: Effects of wortmannin and pertussis toxin. THP-1 cells were pretreated for 16 h with 100ng/ml pertussis toxin, or 15 minutes with 50nM wortmannin, and then $1x10^7$ cells were stimulated with 10nM MCP-1 for various times. Cells were lysed, and lysates were subjected to immunoprecipitation with anti-PI3K-C2β mAb. The washed immunoprecipitates were analysed for PI3Kactivity using PI as a substrate. Extraction and TLC separation of the lipid products were performed as described under 'Materials and Methods'. Lipids were detected by exposure to film at -70°C. Results are representative of four separate experiments.

Figure 4.19 Verification of SHIP expression in THP-1 cells

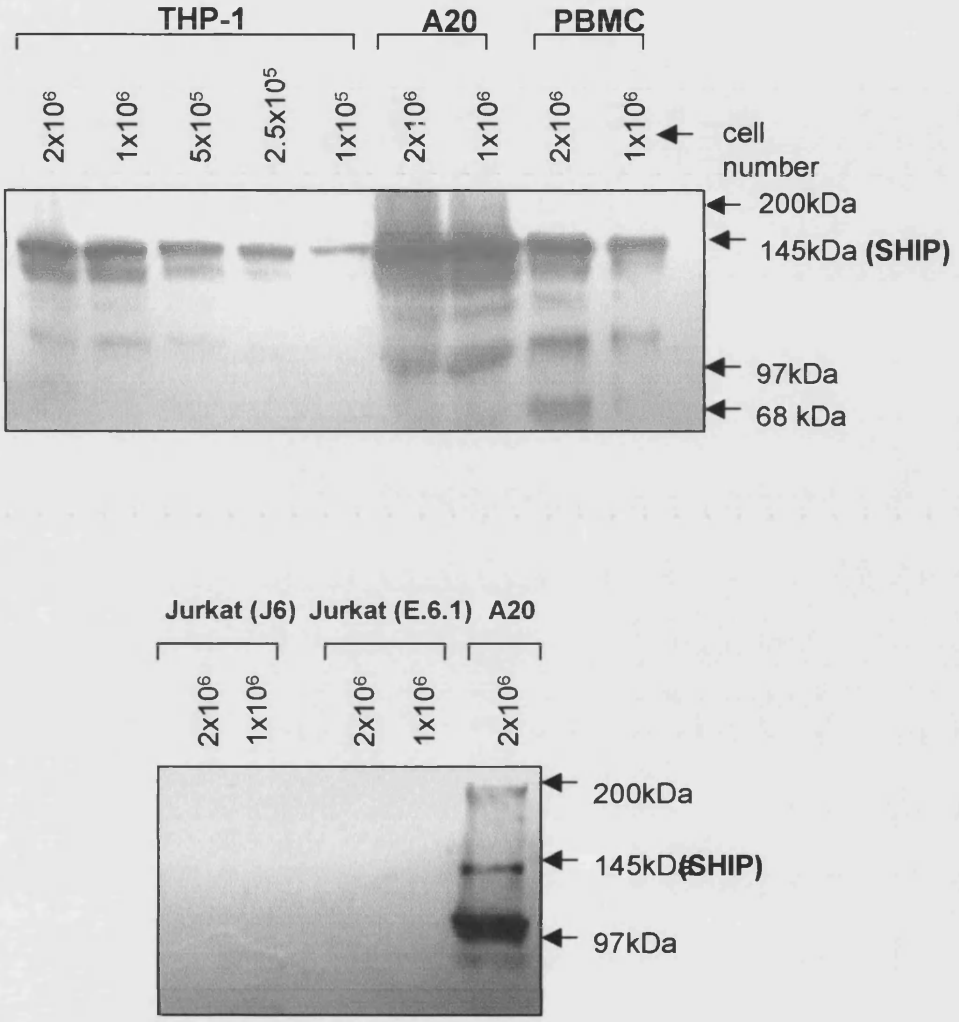


Figure 4.19 Verification of SHIP expression in THP-1 cells. 1 x 10^5 - 2x10^6 cell equivalents of total cell lysates were prepared from non-activated THP-1 cells. Lysates were resolved separately by SDS-PAGE and electrophoretically transferred to a nitrocellulose membrane as described in 'Materials and Methods'. Lysates were immunoblotted with anti-SHIP mAb. A20 and peripheral blood mononuclear cells (PBMCs) were used as positive controls **(A)**, whereas Jurkat cell lysates were used as a negative control **(B)**. Results are from one experiment and representative of another 3 independent experiments.

Figure 4.20 Effect of MCP-1 stimulation on SHIP phosphorylation in THP-1 cells

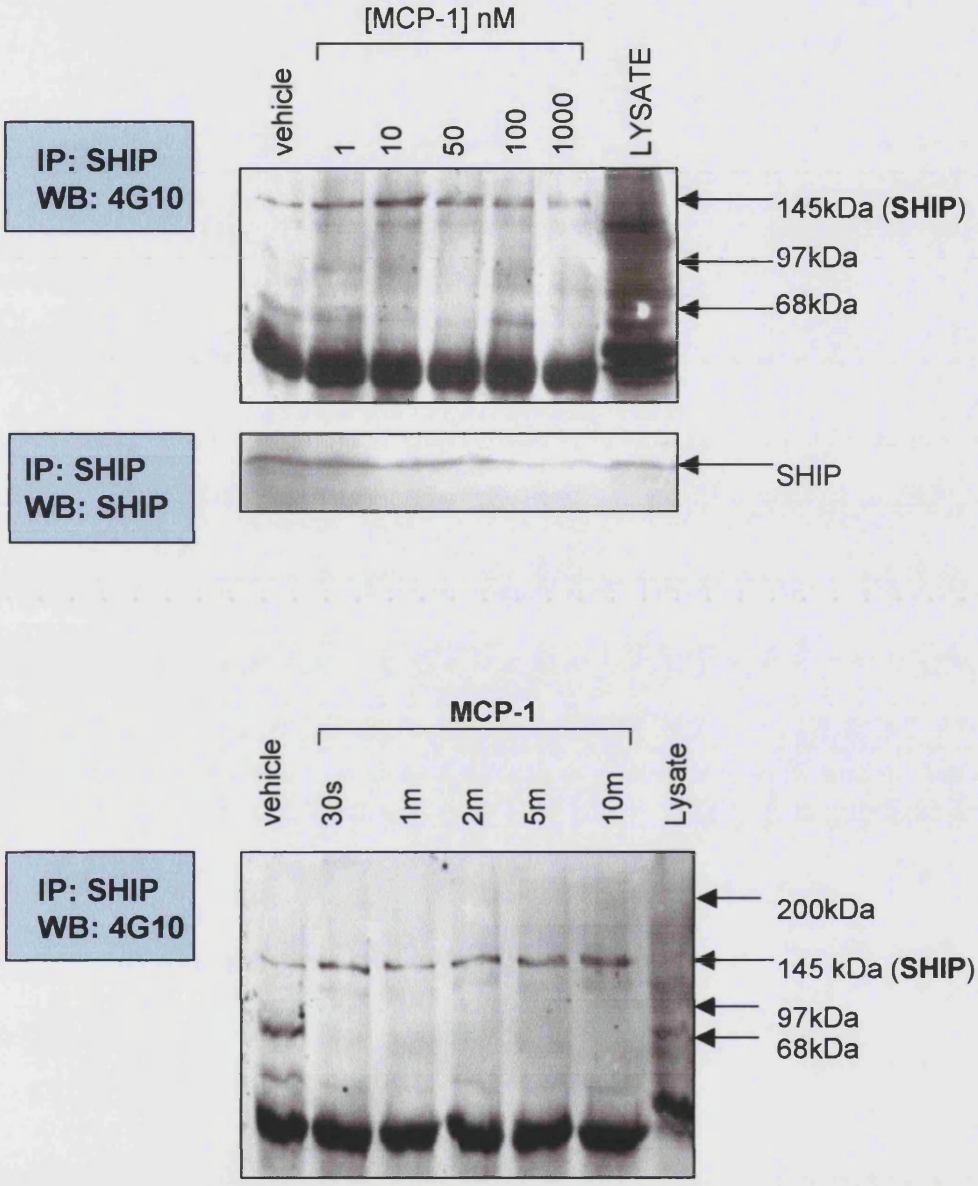


Figure 4.20. Effect of MCP-1 stimulation on SHIP phosphorylation in THP-1 cells. $1x10^7$ THP-1 cells were stimulated with **A)** various concentrations, or **B)** for various times with 10nM MCP-1. Cells were then lysed, and lysates were subjected to immunoprecipitation with anti-SHIP mAb. All samples were resolved separately by SDS-PAGE, electrophoretically transferred to a nitrocellulose membrane and immunoblotted blotted with 4G10 antiphosphotyrosine antibody. Membranes were routinely stripped and reprobed with anti-SHIP antibody to verify equal loading of proteins.

*The western blot shown in A) is representative of at least three experiments, whereas B) is preliminary data that has not been repeated.

Figure 4.21 Effect of wortmannin on MCP-1 and MCP-2-induced chemotaxis of THP-1 cells

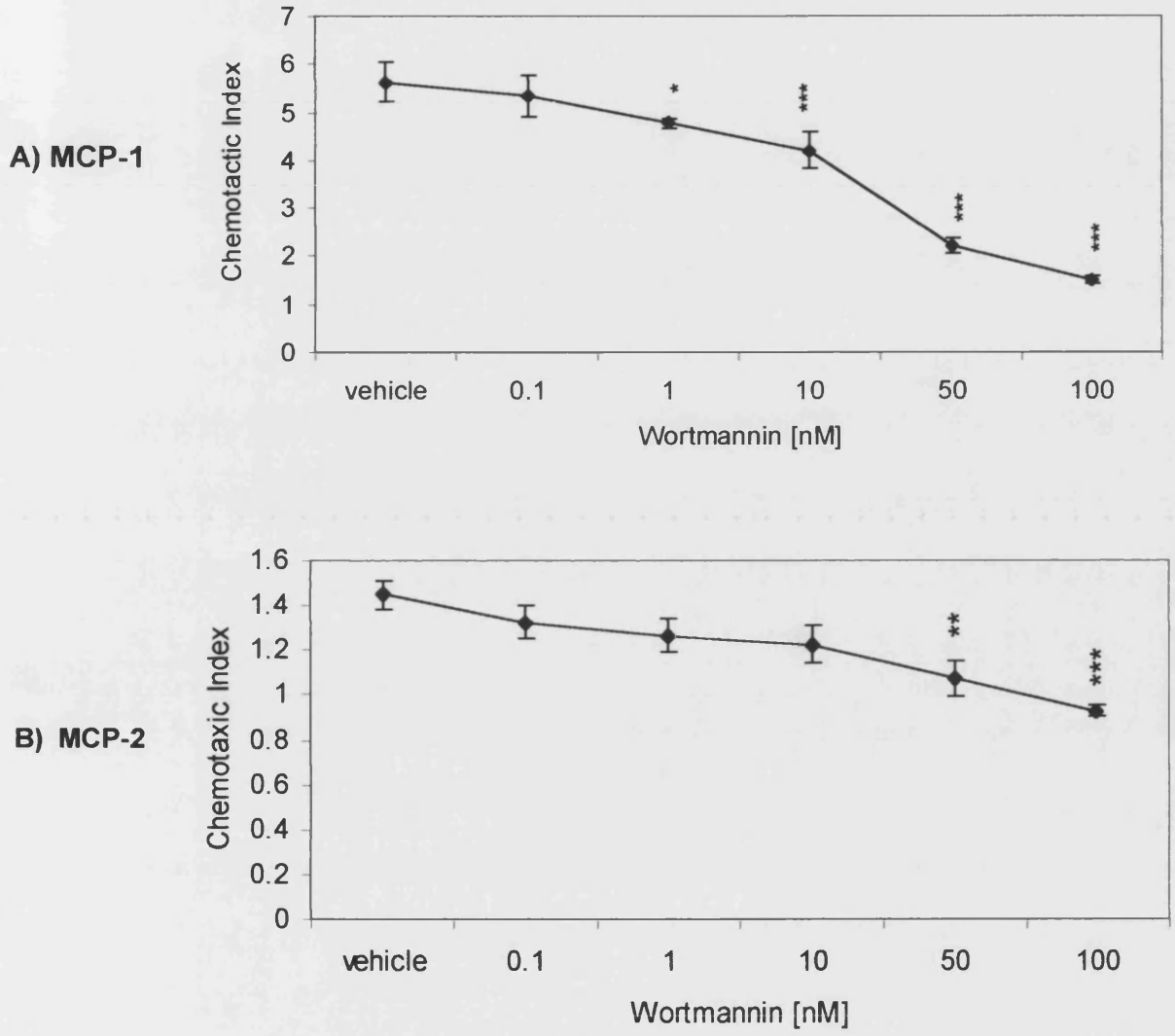
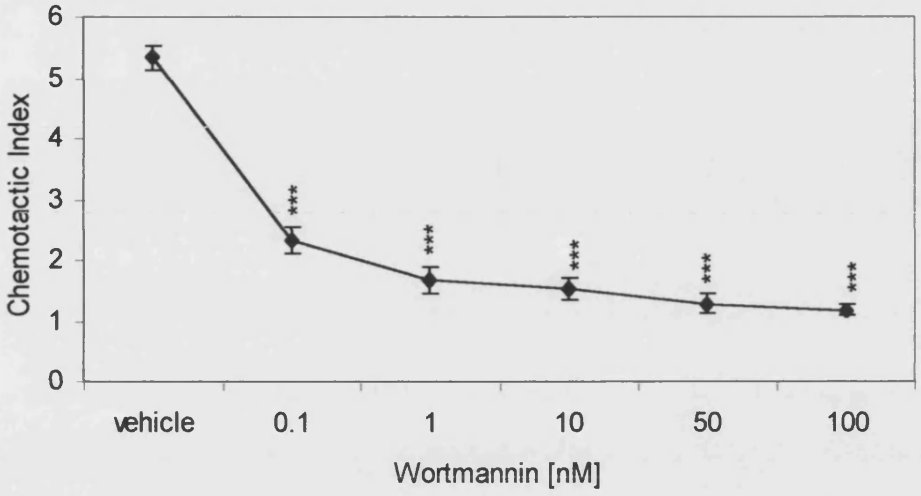


Figure 4.21. Effect of wortmannin on MCP-1 and MCP-2-induced chemotaxis of THP-1 cells. 1 x 10^5 THP-1 cells per point were pre-treated for 15 minutes at 37°C with either vehicle (0.01% DMSO) or various concentrations of wortmannin. After incubation, cells were subjected to chemotaxis towards **A)** MCP-1(1nM) or **B)** MCP-2 (100nM) in a 96-well Neuroprobe™ chamber for 3 hours. Migration was determined as described in 'Materials and Methods'. Results are expressed as a Chemotactic Index (C:I): the ratio of stimulated over basal migration. Data represent the mean ± SEM (5 replicates per chamber/2 chambers per experiment). Results from one experiment but are representative of at least three other independent experiments. *:$p<0.05$, **:$p<0.01$, ***$p<0.001$.

Figure 4.21 Effect of wortmannin on MCP-3 and MCP-4-induced chemotaxis of THP-1 cells

C) MCP-3



D) MCP-4

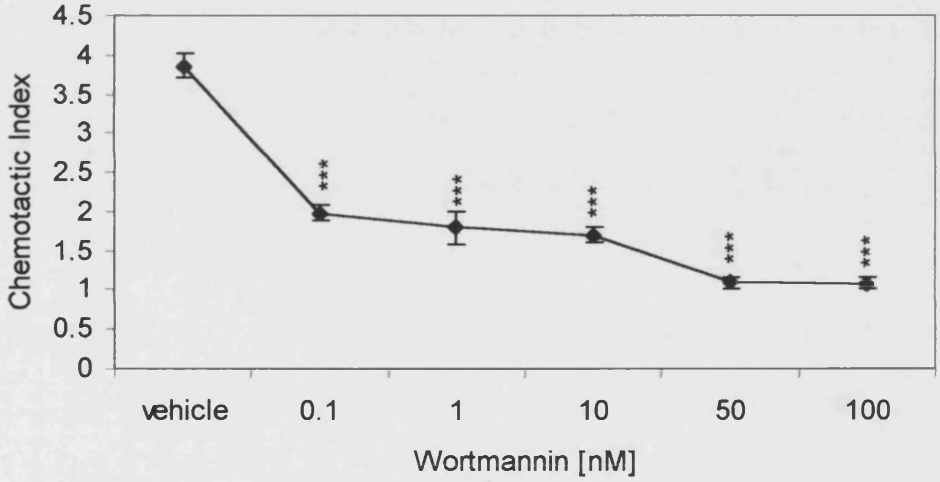


Figure 4.21. Effect of wortmannin on MCP-3 and MCP-4-induced chemotaxis of THP-1 cells. 1 x 10^5 THP-1 cells per point were pre-treated for 15 minutes at 37°C with either vehicle (0.01% DMSO) or various concentrations of wortmannin. After incubation, cells were subjected to chemotaxis towards **C)** MCP-3 (10nM) or **D)** MCP-4 (100nM) in a 96-well Neuroprobe™ chamber for 3 hours. *Migration was determined as described in 'Materials and Methods'*. Results are expressed as a Chemotactic Index (C:I): the ratio of stimulated over basal migration. Data represent the mean ± SEM (5 replicates per chamber/2 chambers per experiment). Results from one experiment but are representative of at least three other independent experiments. *:$p<0.05$, **:$p<0.01$, ***$p<0.001$.

Section 4: Discussion

Activation of PI 3-kinase by CCR2 ligands

Rationale

Many signalling pathways converge on and regulate the PI3K enzymes whose liberation of inositol lipid products provide key mediators of intracellular signal transduction. Different PI3K isoforms generate specific lipids that bind to FYVE and PH domains in an array of proteins, which have consequences on their conformation, localisation and biochemical activities. The activation of PI3K has been reported in a number of systems in response to a variety of agonists that activate both tyrosine kinase -coupled and G-protein- coupled receptors (Mirmonsef *et al* 1999; Montaner *et al*, 1999). Recent work has highlighted the involvement of PI3K in the regulation of chemokine-mediated responses, including cytoskeletal reorganisation, integrin upregulation, and chemotaxis (Curnock *et al*, 2002). Moreover, the differential regulation of these responses by the various isoforms of PI3K is becoming an area of intense investigation. Many examples given in this discussion come from data gleaned from the SDF-1 and its cognate receptor, CXCR4. SDF-1 is one the most extensively studied chemokines with regards to signal transduction mechanisms. This is partly due to the fact that it binds exclusively to CXCR4, and therefore does not exhibit any promiscuity towards other receptor.

The same cannot be said for ligands of CCR2 since all four of them are known to bind to other CC chemokine receptors, however, this receptor is also widely studied in terms of signal transduction, and has provided with a strong foundation from which to launch our investigations. In 1998, Turner *et al* reported that MCP-1 activates the class I p85/p110 PI3K and the class II PI3K-C2α. Here, we have extended these investigations to MCP-2, -3 and -4 and have attempted to further elucidate the relative roles of PI3K isoforms to cellular function.

The two main procedures used in this study to measure PI 3-kinase activity involved measurement of lipid kinase activity in intact cells or broken cell lysates, and both relied on the detection of the transfer of the γ-phosphate of ATP to the D-3 position of the inositol head group of the phosphoinositide lipids. The measurement of kinase

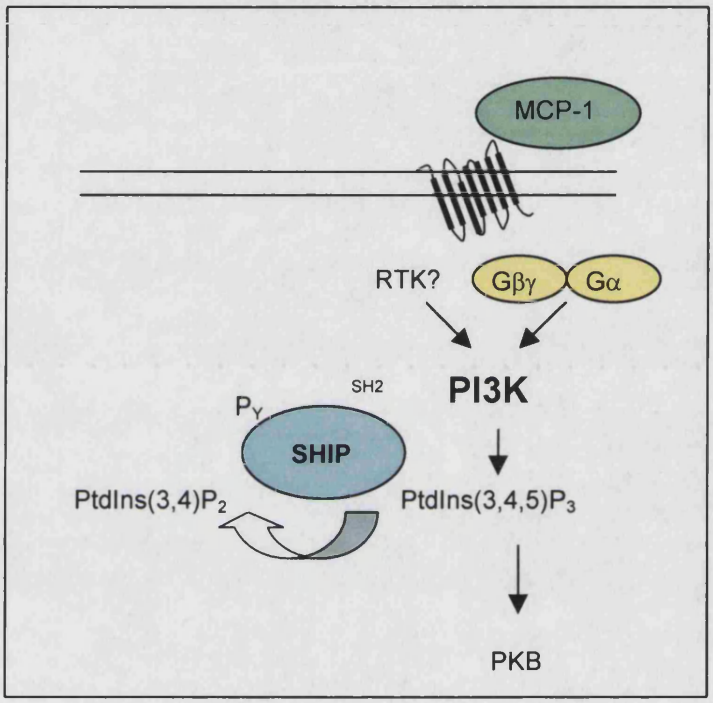
activity in intact cells relied on the metabolic lipid labelling of the cellular pools of ATP with [^{32}P]Pi followed by lipid extraction, separation of phosphorylated lipids by HPLC analysis. For the purposes of this study, we were mainly interested in the accumulation of PtdIns(3,4)P_2 and PtdIns(3,4,5)P_3 in chemokine stimulated THP-1 cells. Overall the kinetics of D-3-lipid accumulation in response to MCP-1, -2, -3 and –4 followed similar trends. All of the chemokines induced a concentration and time-dependent accumulation in PtdIns(3,4,5)P_3 and PtdIns(3,4)P_2. Whereas the rise in PtdIns(3,4,5)P_3 was rapid and transient, the profile of PtdIns(3,4)P_2 demonstrated a delayed yet more sustained rise. It is quite possible that the sustained accumulation of PtdIns(3,4)P_2 was owed the action of a 5-phosphatase that contributed to the generation and breakdown of PtdIns(3,4)P_2 and PtdIns(3,4,5)P_3 respectively. This hypothesis was confirmed by the identification and characterisation of SHIP in THP-1 cells. SHIP is 145kDa inositol-5-phosphatase containing an Src homology 2 (SH2) domain, 2 phosphotyrosine binding (PTB) motifs, and a COOH-terminal proline-rich region. This phosphatase selectively hydrolyses the 5'-phoshphate from both inositol 1,3,4,5-tetraphopshate (IP_4) and PtdIns(3,4,5)P_3 (Damen *et al*, 1996; Lioubin *et al*, 1996).

Several cytokines have been shown to induce tyrosine phosphorylation of SHIP, which may subsequently associate with Shc, the tyrosine phosphatase SHP-2, and Grb2. The actions of SHIP can be attributed, (at least in part) to its capacity to hydrolyse PtdIns(3,4,5)P_3 and restrain the PI3K pathway. By down-regulating PI3K, SHIP has also been shown to inhibit the activation of PKB and Btk, and subsequently has been strongly implicated in the negative signalling in a number of haematopoietic cell models (Liu *et* al, 1997). Given the crucial role of PI 3-kinase in chemokine signalling (Curnock *et al*, 2002) it stands to reason that SHIP activation would play a role in cellular responses to chemokines. Kim *et al* demonstrated that SHIP deficient cells exhibited an enhanced migration towards SDF-1 and BLC. SHIP$^{-/-}$ cells were also more efficient in calcium mobilisation and actin polymerisation in response to SDF-1. The observation of enhancement of chemotaxis of SHIP$^{-/-}$ cells versus wild-types was greater in thymocytes than splenocytes suggested that SHIP-modulated chemotaxis is dependent on the cell type, the receptor and the chemokine studied. In this context, it has been documented that targeted disruption of PI3K is more evident in B cells than T cells. (Fruman *et al*, 1999)

MCP-1 induced a concentration-dependent, and time-dependent tyrosine phosphorylation of SHIP that appeared to correlate with the observed kinetics of the

D-3-lipids. It would have been interesting to further characterise the role of SHIP in CCR2 signalling pathways, perhaps by co-precipitation studies for Shc, SHP-2 or other downstream associates. From our preliminary studies, we are confident that in THP-1 cells, the activation of SHIP by MCP-1 would be in place to restrain the PI3-K/PKB pathway and subsequent functional responses.

Figure 4.22. The role of SHIP in D-3-lipid profiles



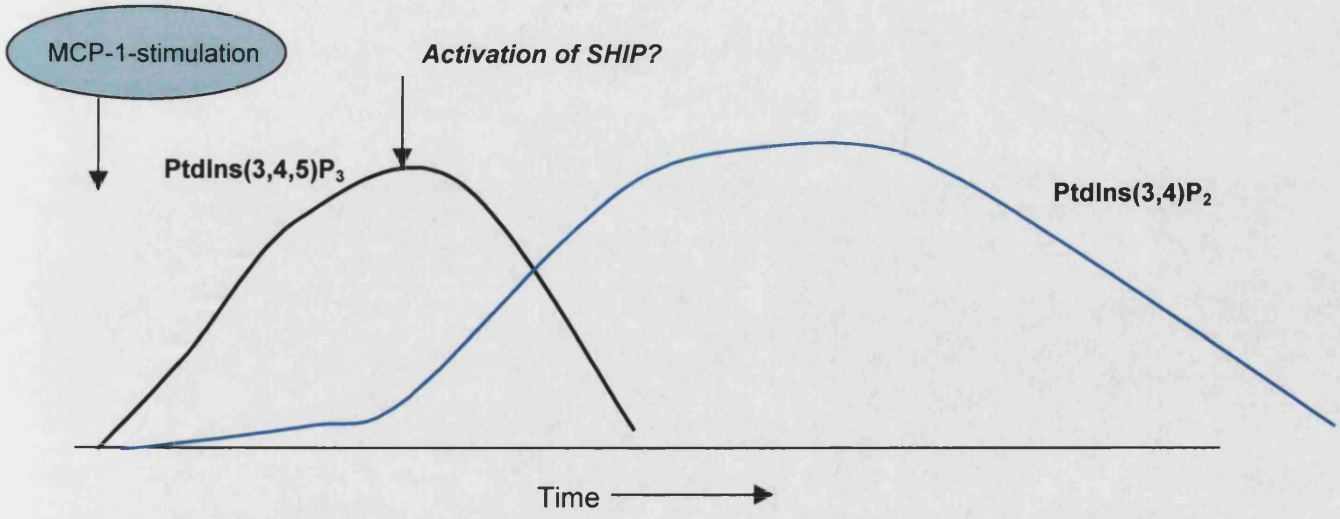


Figure 4.22 The observed differences in kinetics between PtdIns(3,4,5)P_3 and PtdIns(3,4)P_2 accumulation could be owed to the phosphorylation and activation of 5-phosphatase, SHIP. See text for details

In retrospect, it would have been useful to explore the role of other negative regulators of PI3-kinase signalling. PTEN is a tumour suppressor protein that shows homology to protein tyrosine phosphatases and tensin has and has been implicated in the biology of a diverse range of cancers (Maehama and Dixon, 1998). As a dual-specificity phosphatase, it has control over the PI3-K/PKB pathways by dephosphorylating D-3-lipids on the 3'-phosphate from the inositol ring, thus, converting PtdIns(3,4)P_2 and PtdIns(3,4,5)P_3 to PtdIns(4)P and PtdIns(4,5)P_2 respectively. In the context of chemokine signalling, PTEN has been shown to inhibit cell migration by dephosphorylating key tyrosine-phosphorylated proteins, thereby antagonising interactions of integrin-triggered signalling pathways (Tamura *et al*, 1999). The strong interdependency between PI3-K/PKB pathways and lipid phosphatases are becoming increasingly apparent. A recent study in *Drosophila* demonstrated the paramount importance of PKB in mediating the effects of PIP_3 concentrations in PTEN deficiency, and confirmed that PKB appears to be the only critical target activated by increased levels of PIP_3. It was further concluded that flies devoid of PTEN could survive abnormally high concentrations of PtdIns(3,4,5)P_3 if the affinity of PKB for PtdIns(3,4,5)P_3 (via the PH domain) is decreased (Stoker *et al*, 2002).

In our experiments, the elution peaks for PtdIns(4)P and PtdIns(4,5)P_2 were not consistent enough to extrapolate any data for these lipids. It would have been interesting to assess the profiles of these products and perhaps ascertain a role for a 3'-phosphatase as well as the 5'-phosphatase, SHIP, in the regulation of this pathway. Preliminary studies showed that PTEN was highly expressed in the THP-1 cell line (data not shown), but due to the boundaries of this project, investigations were not taken any further.

Activation of multiple PI3K isoforms by CCR2 ligation

Although measurement of D-3-lipid accumulation and the effects of pharmacological inhibitors demonstrated that PI3K was indeed an important signalling molecule in this system, these experiments provided limited information with regards to the specific isoforms of PI3K that were being activated. This is where the second method of PI3K measurement was employed. Here, we assayed specific immunoprecipitated PI3K subunits for associated lipid kinase activity under *in vitro* conditions using PtdIns as a substrate. The application of *in vitro* lipid kinase assays enabled us to identify the involvement of specific PI3K isoform in response to CCR2 ligation. As

expected, activation of GPCR-specific isoforms, p110γ, was observed in response MCP-1, -2, -3, and –4, and in the case of MCP-1, this activity was abrogated with pertussis toxin and wortmannin. Considering that chemokine receptors are by nature G protein-coupled, it would be reasonable to implicate PI3Kγ in mediating accumulation of PtdIns(3,4,5)P_3 – as has been previously observed in SDF-1-stimulated Jurkat cells (Sotsios *et* al, 1999). However, although this response was abrogated by pertussis toxin, the resistance of MCP-1-stimulated PtdIns(3,4,5)P_3 accumulation to LY290042 makes this an unlikely hypothesis. Nevertheless, PI3Kγ as a key mediator of D-3-lipid accumulation has been observed in similar systems. Interestingly, leukocytes from genetically modified mice with a PI3Kγ-deficient phenotype are unable to produce PtdIns(3,4,5)P_3 in response to the CXC chemokine, IL-8 (Hirsch *et al*, 2000).

The kinetics of p110γ activation in response to MCP-1 demonstrated an interesting pattern. The time-course experiments implied that there was a biphasic activation of this isoform which peaked at around 30 s followed by a second peak at approximately 10-15 m post-stimulation. Furthermore, this observation correlates with a similar study with IGF-1-stimulated T cells where by PI3K and PKB activity followed a biphasic pattern (Hershberg and Mayer, 2000). It is possible that this activation profile with characteristic early and late functions denotes that PI3K could be capable of supporting immediate and sustained cellular responses in response to MCP-1 stimulation.

Although there is a wealth of strong biochemical and genetic evidence for the activation of PI3Kγ by chemokines, it is becoming increasingly clear that this may not be the only isoforms capable of transducing chemokine-stimulated responses. This comes largely from the observation that in PI3Kγ deficient mice there was only a 50-70% inhibition of neutrophil chemotaxis (Hirsch *et al*, 2000; Li *et al*, 2000; Sasaki *et al*, 2000). It is possible that other isoforms are activated by these receptors. Indeed, *in vitro* lipid kinase activity has been observed in p85 immunoprecipitates in response to SDF-1 and RANTES in T cells (Turner *et al*, 1995: Sotsios *et al*, 1999) and by MCP-1 in THP-1 cells (Turner *et al*, 1999).

Activation of the prototypical p85/p110 heterodimer was seen in response to MCP-1, and confirmed previous observations in this system in that it was a wortmannin-sensitive, time-dependent event. However, it seems unlikely that the p85/p110

heterodimer would be significantly contributing to overall detectable D-3 lipid pool stimulated by MCP-1, -2, -3 or –4 due to the apparent resistance to wortmannin with regards to lipid accumulation. Our investigations into the role of this isoform did not go beyond this point, although it would have been useful to have identified any involvement of tyrosine kinases in D-3-lipid accumulation. Such studies have been carried out in SDF-1-stimulated Jurkat cells: Here, the p85/p110 heterodimer was not suspected to be instrumental in the generation of D-3-lipid products on account of its sensitivity to pertussis toxin and resistance to Herbimycin-A. This pharmacological evidence was further confirmed using U937:Δp85 cells, whereby the loss of coupling to p110α,β,γ isoforms by Δp85 had no effect on SDF-1-stimulated PtdIns(3,4,5)P_3 (Sotsios *et al*, 1999)

Previous studies have also shown that in THP-1 cells, MCP-1 induces lipid kinase activity to co-associate with anti-phosphotyrosine immunoprecipitates, and thus further evidence that the prototypical p85/p110 heterodimer was being activated in a phosphotyrosine-dependent manner. This has also been observed in CXCR4-expressing pre-B cell lines in response to SDF-1 stimulation (Ganju *et al*, 1998), as well as in MCP-1-stimulated THP-1 cell.

The observation of protein tyrosine phosphorylation has been observed in response to a number of chemokines, however, the assessment of tyrosine phosphorylation in response to MCP-1, -2, -3 and –4 stimulated THP-1 cells was a crucial set of experiments that failed to be carried out in this study. Although our group has previously shown that MCP-1-induces tyrosine phosphorylation of proteins at 50, 80 and 120kDa in THP-1 cells, it would have been useful to out investigations to compare this against other CCR2 ligands.

How would CCR2 couple to the class I_A p85/p110 heterodimer?

Coupling of p85/p110 PI3K is know to require interaction of the SH2 domains within the p85 regulatory subunit with specific phosphotyrosine-containing binding motifs (pYXXM; where pY represents phospho-tyrosine) located in growth factor receptors and adapter molecules (Ward *et al*, 1996). The mechanism by which chemokine receptors associate with p85/p110 is ill-understood. Several groups have proposed that the GPCR-mediated activation of p85/p110 PI3K is activated by the βγ subunits. However, the caveat with this theory is that few chemokines are known stimulate events of tyrosine phosphorylation – and the p85/p110 is known to co-associate with

phosphotyrosine-containing proteins (Stephens *et* al, 1997; Vanhaesebroek *et al*, 2000). A more feasible route by which chemokine receptors could regulate p85/p110 in a phosphotyrosine-dependent mechanism, could be through modulation of the $G\alpha_i$. This comes from the observation that GTP-bound $G\alpha_i$ subunits can bind and activate Src and Hck (Ma *et al*, 2000). In terms of linking to the class I PI3K pathway, the association of $G\alpha_i$ may also extend to other members of the Src kinase family (e.g. Lck and Lyn) as has been suggested for the GPCR-mediated route to Ras activation (Ganju *et al*, 1998).

Class II PI3K: A novel class of PI3K activated by CCR2

In our system, the observation that the pertussis toxin-sensitive accumulation of PtdIns(3,4,5)P_3 was in fact resistant to PI3K inhibitors provided evidence that neither the p85/p110 heterodimer nor the PI3Kγ was involved in this response. We were subsequently led to observe the effects of MCP-1 on the novel class II PI3Ks, PI3K-C2α and PI3KC2β, which have been shown to have a reduced sensitivity to wortmannin (Domin *et al*, 1997). Recent studies have shown that like the class I enzymes, class II PI3Ks also act downstream of receptors for growth factors (Wheeler and Domin, 2001) and for chemokines (Turner *et al*, 1998) and for integrins (Zhang *et al*, 1998). Here we have shown that MCP-1 activates PI3-KC2α and PI3-KC2β in THP-1 cells in a time-dependent manner, albeit with slightly different kinetics.

Assuming that these class II enzymes are responsible for the observed MCP-induced PtdIns(3,4,5)P_3 accumulation, we have a number of hypotheses as to how they may be involved in CCR2-mediated signal transduction. From analogy with what has been observed about GPCR coupling to PI3Kγ, there are at least two putative mechanisms by which MCP-1 could activate PI3K-C2α/β in a pertussis toxin-sensitive manner: (i) direct interact of G$\beta\gamma$ subunits with PI3K-C2α/β as has been reported to occur for PI3Kγ (Leopoldt *et al*, 1998); (ii) G$\beta\gamma$ subunits may activate PI3K-C2α via protein tyrosine kinases considering that there is evidence supporting PTKs in $G_{i/0}$-mediated cell signalling (Ptasznik *et al*, 1995; Lopez-Ilasca *et al*, 1997).

It could follow that the pertussis toxin-sensitive MCP-1-induced activation of PI3K-C2 α/β, maybe solely or partly be responsible to the detectable changes in PtdIns(3,4,5)P_3. Interestingly, in contrast to other reports, our data show that there is in fact a moderate wortmannin-sensitivity in PI3K-C2β immunoprecipitates, and this

observation has recently been confirmed in Jurkat cells (K.Patel, unpublished observations). It may possible that PI3K-C2α is the key mediator in D-3-lipid accumulation in MCP-1-stimulated THP-1 cells. However, caution must be taken when considering this theory since the *in vitro* substrate specificity of PI3K-C2α is restricted to PtdIns and PtdIns(4)P (Domin *et al*, 1997), and would apparently contradict it's potential role in mediating PtdIns(3,4,5)P_3. That said, it is conceivable that the substrate specificity of this isoforms may be quite different in intact cells to that reported under *in vitro* conditions.

Further clues from the downstream effectors of PI3K

We extended our investigations of the PI3K pathway by testing the involvement of protein kinase B (PKB), a kinase that is activated downstream of PI3K, with the participation of other signalling molecules such as PDK-1 (for review: Coffer and Woodgett, 1999). Several G protein coupled receptors, including those activated by chemokines have been shown to activate PKB in a PI3K-dependent manner (Sotsios *et al*, 1999; Turner *et al*, 1998), and this is supported by the observation that IL-8 is unable to stimulate PKB in P3Kγ-deficient neutrophils (Hirsch *et al*, 2000). Emerging evidence has implicated PKB activation as a prerequisite for efficient chemotaxis in *Dictyostelium* (Meili *et al*, 1999). Inhibition of PI3Ks with wortmannin or LY290042 blocks chemoattractant-induced translocation of PH-PKB green fluorescent protein and subsequent activation of PKB (Tilton *et* al, 1997). However, although cytoskeletal reorganisation and lamellipodium are events mediated by PI3K (Cox *et al*, 1999), they can occur independently of PKB (Ma *et al*, 1998).

In our system, MCP-1, -2, -3 and –4 induced a transient increase in PKB phosphorylation, thus confirming that the PI3K/PKB axis is a target of CCR2 signalling. Interestingly, whereas manumycin had no effect on D-3-lipid accumulation, treatment with wortmannin almost completely ablated PKB activity. This apparent discrepancy in biochemical output is difficult to explain. Since the favoured substrate of PKB is PtdIns(3,4,5)P_3, and to a lesser extent, PtdIns(3,4)P_2, it is unlikely that any other lipid could be modulating PKB activation. So, if indeed PtdIns(3,4,5)P_3 and PtdIns(3,4)P_2 are responsible for it's activation, why does wortmannin block the activity of PKB, but not its substrates? It is possible that although gross accumulation of PtdIns(3,4,5)P_3 and PtdIns(3,4)P_2 in the overall lipid pool can be measured *in vivo*, a small amount lipids generated by wortmannin-sensitive PI3K isoforms could go undetected – and it is these residual D-3-lipids that may account for the wortmannin-sensitive PKB activation observed in our system.

Given the role of PKB as a key player of growth factor-induced cell survival and protection, identification of the substrates of PKB is subject to intensive research, since at least one must play a role in the suppression of apoptosis. Therapeutic targeting strategies have progressed from inhibition of the PI3K/PKB axis, to the identification, and possible manipulation of the negative regulators of PKB. For example, the recently identified CTMP (carboxy-terminal modulator protein) inhibits phosphorylation of PKB by binding to the COOH-regulatory domain of PKBα. One of several mechanisms for preventing inappropriate kinase activation and subsequent excess proliferation, and thus, has potential implications for cancer biology (Maira *et al*, 2002).

The principle physiological substrate of PKB is glycogen synthase kinase-3 (GSK-3), which was initially identified as an enzyme that regulates glycogen synthesis in response to insulin (Pap and Cooper, 1998). The demonstration that stimulation of THP-1 cells with MCP-1, -2, -3 and -4 resulted in phosphorylation of GSK-3 was further evidence that the PI3K/PKB pathway is a critical element of CCR2-mediated signal transduction. Interestingly, this is not the first chemokine to have been shown to activate this pathway. For example, the TECK/CCR9 interaction activates GSK-3 and the forkhead transcription factor (FHKR), and provides a cell survival signal to receptor expressing cells (Youn *et al*, 2001).
It was show in these studies that MCP-1, -2, -3 and –4 phosphorylate GSK-3 in a comparative fashion to that of PKB. However, in the case of MCP-2-stimulated THP-1 cells, there was a lack of correlation in THP-1 cells in terms of the sensitivity of GSK-3 to wortmannin. Although reduced, GSK-3 was activated by MCP-2 even in the presence of wortmannin suggesting that a PKB-independent route may activate GSK-3. and further underscores the multiple levels of cross-talk among the PI3K signalling cascades in this system.

There is growing evidence of mechanisms of GSK-3 phosphorylation and inactivation independent of a functional PI3K-PKB pathway. For example, such modes have been previously demonstrated in the Wnt pathway required for *Drosophila* development (Miller and Moon, 1996). More recently Fang *et* al, have shown that GSK-3α/β are also physiological substrates of cAMP-dependent protein kinase A (PKA). Here, PKA physically associates with, phosphorylates, and inactivates both isoforms of GSK-3. Thus PKA functions as a GSK-3 kinase that, in parallel with PKB, controls the activity of the multifunctional enzyme GSK-3. In this example, the

convergence of the PKA and PKB signalling pathways at GSK-3 suggests that, depending on the environmental context, the activity of GSK-3 can be regulated either by growth factors (e.g., insulin and IGF-1) working through the PI3K-PKB cascade or by hormonal stimulation (e.g., adrenaline) of G protein-coupled receptors that link to changes in intracellular cAMP concentrations (Fang *et al*, 2001).

PI3K is a key modulator of cell migration

Having demonstrated that engagement of CCR2 activates a number of PI3K isoforms, it was important to determine the relative role of this enzyme on functional output. For some time, PI3K has been considered to be an important regulator of cell migration in many systems including those elicited by chemokines. Elegant molecular and pharmacological evidence first suggested that PI3K and its lipid products play a significant role in PDGF-dependent actin polymerisation and cell migration (Kundra *et* al, 1994). As discuss in Section 5, the Rho GTPases (Rho, Rac and Cdc42) are regulators of actin cytoskeleton and cellular polarity and there is much interest in ascertaining their relationship with PI3K-dependent signalling. Indeed, there is overwhelming evidence to indicate that Rho family kinases are regulated by PI3K is many systems (Hall *et al*, 1998; Tolias *et al*, 1995). Other studies have pinpointed the specific PI3K subunits necessary for such regulation. Jiminez *et* al have described how PDGF indirectly activates Cdc42 and further proposed that the regulatory subunit of PI3K, p85α, is one of the molecules controlling this process. In this respect, it was postulated that p85α performs as an adapter molecule by bringing a GTPase regulator into the complex that mediates Cdc42 activation.

The motility of the cell not only requires actin polymerisation, but also the modulation of cell polarisation that coordinates adhesion at the leading edge and contraction at the trailing edge. PI3K is just one of an emerging number of signalling molecules that have been heavily implicated in leukocyte polarisation in cell migration and immune interactions (Sanchez-Madrid *et al*, 1999). Chemokines can also activate PI3K-dependent signalling cascades implicated in the regulation of chemotaxis and modulation of integrin affinity (Kundra *et al*, 1994; Hartwig *et al*, 1995). This is highlighted by the observation that RANTES-induced T cell chemotaxis and polarisation is abrogated by PI3K inhibitors (Turner *et al*, 1995). Gerszten *et al*, have recently shown that PI3K are necessary for chemokine-triggered conversion of monocyte rolling to firm adhesion under physiological flow conditions

In this system, it is clear that PI3K plays a fundamental role in the migration of THP-1 cells towards MCP-1, -2, -3, and –4. However, with the exception of MCP-2-induced chemotaxis (which was totally abrogated by wortmannin), a residual level of migration is still observed even in the presence of high concentrations of PI3K inhibition. This is suggestive that cell migration in this system is mediated by other PI3K-independent mechanisms. Given that we have identified a number of isoforms activated by engagement of CCR2, it would have been interesting to elucidate which of these were responsible for migration. Although p110γ has been heavily implicated in chemokine-mediated responses, it is unlikely that such complex processes are entirely dependent on one isoform. It is more likely that there is an integration of several signalling events that could be provided by more than one of the identified PI3Ks. Several lines of evidence are indicative that although p85/p110 heterodimer does not contribute to SDF-1 or MCP-1-stimulated D-3-lipid accumulation (Sotsios *et al*,1999; Turner *et al*, 1998) , it may still have a functional relevance. For example, this isoform could perhaps account for the residual neutrophil chemotaxis observed in p110γ-deficient mice.

As suggested from our lipid data and PKB activation profiles, it is possible that the wortmannin-sensitive p85/p110 heterodimer could have a small, undetectable but nevertheless significant role in the contribution to the overall D-3-lipid pool. Hence, this isoform could be providing an important role in CCR2-mediated cell migration. It must also be considered that physiological role for PI3K may reside in its protein serine kinase activity rather than it lipid kinase activity. It is important for future studies to establish the potential for bifurcation of lipid and protein kinase signals to distinct downstream effectors (Bondeva *et al*, 1998).Although we have suggested the possible involvement of PI3K-C2α and PI3K-C2β in this system, as of yet there is no information as to whether their activation is of functional significance. For this we await the development of selective pharmacologicalal inhibitors, and strategies for genetic modification.

Activation of multiple PI3Ks: A functional strategy?

As we have demonstrated in our studies, activation of more than one PI3K can ensue chemokine receptor ligation. There is incredible potential for heterogeneity of chemokine receptor isoforms and/or G protein subunits that may give rise to differential regulation of PI3K isoforms. Furthermore, the contribution of PI3K isoforms to certain signalling events can also vary according to the cell type and receptor studied. For example, although CCR2A and CCR2B can couple to the G_i-

$G\beta\gamma$-PLCβ2 pathway, their splice variants exhibit a unique specificity in coupling to the α subunits of the G_q class. In this respect, CCR2 is able to couple $G\alpha_{16}$ and $G\alpha_{14}$, whereas CCR2A cannot couple to either of these (Kuang *et al*, 1996). Given the potential diversity within the coupling of chemokine receptor to G protein subunits and PI3K isoforms, it is no surprise that chemokines have the ability to elicit such a diverse range of functional responses. The differential regulation of signalling pathways through chemokine receptors permits leukocytes to have concurrent control of adhesion molecules regulation, cytoskeletal reorganisation, shape change, granule release and superoxide release.

Future Investigation

- Extend the investigation of the PI3K isoforms activated by MCP-2, MCP-3 and MCP-4 in comparison to MCP-1. Is there differential regulation of PI3K isoforms via CCR2 ?

- Co-precipitation studies: Investigate the molecular associations of PI3K subunits. Examine the formation of PI3K-related signalling complexes ensuing CCR2 engagement.

- Carry out comprehensive screening of tyrosine phosphorylation events elicited by CCR2 ligation. Assessment by 1) western blotting, and ; 2) the *In vitro* lipid kinase activity of 4G10 immunoprecipitates derived from MCP-stimulated THP-1 cells.

- Look at the effects of inhibitors of tyrosine phosphorylation on MCP-induced accumulation of D-3-lipids, and *in vitro* lipid kinase activity of immunoprecipitated PI3K isoforms.

- Using GFP-tagged PKB constructs: Observe the cellular redistribution of PKB and the role of its PH domain following CCR2 engagement.

Section 5: Results

The Role of PI 3-kinase in ERK-1/2 activation

Rationale

As discussed in Section 4, PI3-kinase is a critical enzyme for multiple cellular functions and may be activated by several pathways, depending on the isoform involved and the regulatory molecule implicated. Cross talk between PI3-kinase and other signalling cascades has been well documented, with the best characterised being that of the PI3-K-Ras/MAPK pathway. Interestingly, for several chemokines it has been shown that receptor binding activates the ERK (extracellular signal related)/MAPK pathway in multiple models including human monocytes and monocytic cell lines (Knall *et al*, 1996; Yen *et al*, 1997; Coffer *et al*, 1998; Ganju *et al*, 1998). Although MCP-1 has been shown to activate several signalling cascades, including the PI3-kinase and ERK/MAPK pathways (Turner *et a*, 1998; Ashida *et al*, 2001), the molecular mechanisms and cross-talk between systems remains ill-understood. Moreover, the ability of other CCR2 ligands (MCP-2, -3 and -4) to activate such systems has not yet been addressed. In this section, we attempt to explore 1) the ability of CCR2 ligands to activate the MAPKs including the SAPKs (stress activated proteins kinases); 2) the role of PI-3K in CCR2-mediated MAPK activation; and 3) the relevance of these signalling cascades to cell migration.

Results

Activation of MAPK cascade

We investigated whether the MCP ligands induced phosphorylation of ERK-1/2, p38, and JNK in THP-1 cells, with phosphorylation being a prerequisite for inducing kinase activity. Accordingly, THP-1 cells were incubated with MCP-1, -2, -3 and 4 using concentration known to elicit an optimal functional response (1nM, 10nM, 10nM and 100nM, respectively) for varying lengths of time, and cell lysates were subjected to immunoblotting using antibodies that specifically recognise the phosphorylated forms of these kinases. In addition, the use of pharmacological inhibitors to G-proteins, PI3-K, PKC and MEK helped to further define the implication of individual signalling molecules to MCP-induced MAPK activation.

MCP-1, -2, -3 and -4 activate MAPKs via a PI3-kinase-dependent mechanism

Figure 5.1. MCP-1-stimulated THP-1 cells showed rapid and transient phosphorylation of ERK-1/2 with maximal activity at 2-5 minutes after stimulation (kinetics varied between experiments), and returned near basal levels after by 10 minutes. Pre-treatment with 50nM wortmannin only partially ablated ERK-1/2 phosphorylation, which indicates that MCP-1-stimulated activity of this pathway is partially dependent on PI3-kinase.

MCP-2, -3 and -4 also stimulated transient increases in ERK-1/2, albeit with slightly different kinetics to that observed with MCP-1. Wortmannin served to completely abrogate MCP-3 and -4 -stimulated ERK-1/2 activation, whereas with MCP-2-stimulated cells, ERK-1/2 phosphorylation was only partially inhibited.

MCP-1, -2, -3 and -4 activate SAPKs via a PI3-kinase-dependent mechanism

In addition to ERK/MAPK activation in THP-1 cells, the effects of CCR2 ligands on stress activated protein kinases (SAPKs), p38/SAPK and JNK/SAPK were also assessed. Activation of p38 requires phosphorylation of Thr-^{180} ad Tyr-^{182} (Lee *et al*, 1994) and can be assayed directly using phospho-specific antibodies. MCP-1 and MCP-3 both transiently activated p38 with maximal activity seen 2-5 min post-stimulation. Pre-treatment with wortmannin also demonstrated that p38 pathway in response to MCP-1 ad MCP-3 is entirely dependent on PI3-K. MCP-2 and MCP-4, demonstrated a lesser dependency on PI3-K since wortmannin served to partially inhibit phosphorylation of p38. MCP-1, -2, and -3 showed similarities kinetics of p38 activation (peaking 2-5 min post-stimulation), however, MCP-4 was maximal at approximately 10 min after stimulation.

In addition, we also investigated the activation of the c-Jun N-terminal stress-activated protein kinase 1/2 (JNK1/2). When activated, JNK1 and JNK2 become phosphorylated on Thr^{183} and Tyr^{185}, respectively, and this was assessed using an anti-dual phosphorylated JNK1/2 antibody. Figure 5.2 demonstrates how all four CCR2 ligands induce activation of JNK1/2 as shown by bands corresponding to 46kDa and 55kDa. Similar to the pattern observed with p38, MCP-1 and MCP-3 rapidly and transiently activated JNK1/2 in a manner that appeared to be entirely dependent on PI3K. MCP-2 and MCP-4-stimulated JNK phosphorylation, on the other hand, was only partially dependent on PI3K. Again, MCP-4 exhibited delayed kinetics in activation of SAPK, showing maximum activation of JNK1/2 after 10 min.

Small GTP binding proteins in MCP-1-induced MAPK activation.

We used a fusion protein (GST-RBD) comprising the Ras-binding domain of Raf to selectively precipitate active Ras, which interacts with this domain of Raf-1 (DeRooij and Bos, 1997). In unstimulated cells, active Ras was clearly detectable as a single band at 21kDa (Figure 5.3). Stimulation of cells with MCP-1 resulted in a greater amount of precipitated activity after 5 m, indicating increased activity. The effects of MCP-2, on the other hand were inconclusive. Stimulation with this chemokine failed to give a clear result. As a positive control, Jurkat cells, stimulated with PMA, induced a detectable increase in Ras activity after 10 m.

The role of Ras in downstream signalling pathways was assessed using the selective bacterial toxins from *Clostridium sordelli*. These virulence factors, known as 'lethal toxins', specifically glucosylates and inhibits small GTPases. Although lethal toxins used in this study have the highest selectivity for Ras, other small GTPases are also modified. LT82 modifies Ras, Rac, Rap and Ral, whereas LT9048 modifies Rac, Cdc42, Rap (weakly) and Ral (very weakly). Figure 5.4 shows the dose-dependent effects of LT-82 on basal activation of ERK-1/2. It was demonstrated that 500ng/ml (2 hour pre-treatment) was the minimum concentration required to inhibit ERK-1/2 activity in this system. Based on these results, this concentration was used in subsequent biochemical experiments.

When incorporated into a time-course experiment, it can be seen that LT-82 completely abrogated MCP-1-stimulated ERK-1/2 activation (Figure 5.5). The densitometric analysis (normalised with equal loading controls) demonstrated that this toxin reduced ERK-1/2 activation to basal levels. LT-9048, on the other hand, reduced constitutive activity of ERK-1/2, but yet did not completely ablate MCP-1-stimulated activation. Although reduced, a signal was detected 2 min post-stimulation.

We then went on to explore the role of small GTPases in PI3K activation. LT-82 served to inhibit MCP-1-induced PtdIns(3,4,5)P_3 accumulation in THP-1 cells as analysed by ^{32}P metabolic labelling of phospholipids (Figure 5.6). Protein kinase B (PKB) has always been recognised as a pathway that lies downstream of PI3K. MCP-1-induced PKB phosphorylation on the activation specific Ser-473, was markedly inhibited to near basal levels with LT-82 and LT-9048 (Figure 5.7). These

results were highly indicative that Ras is involved in the PI3K-mediated accumulation of D-3-phosphatidylinositol lipids in this system. Figure 5.8. *In vitro* lipid kinase assays of immunoprecipitated subunits of PI3K showed the effects of lethal toxin on specific isoforms of PI3K, namely p85/p100 heterodimer and PI3Kγ. In the case of p85, the basal activity of these immunoprecipitates was increased by LT-82, although stimulated activity was attenuated - However, due to the LT-induced increase in basal expression of p85 and subsequent masking of stimulated activity, these results were inconclusive. MCP-1-stimulated activity of the p110γ immunoprecipitates was clearly inhibited by this toxin, and baseline activity was unaffected. Class II PI3Ks were not investigated.

Because we have shown that a) Ras lies upstream of PI3K, and b) cell migration is critically regulated by PI3K in this system, we tested the effects of the lethal toxins on MCP-1-induced chemotaxis. Figure 5.9. Both LT-82 and LT-9048 drastically inhibited cell migration towards MCP-1 (IC_{50} = 3.6 and 11.8 ng/ml, respectively), to below basal levels – thus, indicating a role for small GTPases, such as Ras, in chemokinesis as well as chemotaxis.

CCR2 couples to PTX sensitive and PTX-insensitive pathways

Figure 5.10. Pertussis toxin (PTX) was used to investigate the involvement of specific G-protein subunits in ERK-1/2 activation is response to MCP-1, -2, -3 and –4. As previously established, all four ligands induced a time-dependent increase in ERK-1/2 phosphorylation in the absence of inhibitor. When pre-treated with PTX (100ng/ml, 16 hr), chemokine-stimulated ERK-1/2 activation was severely compromised. In the case of MCP-2, -3, and –4, ERK-1/2 phosphorylation was attenuated, however, in MCP-1-stimulated cells, a small amount of residual signalling was evident at 5 m post-stimulation.

Interestingly, the effect of PTX on JNK phosphorylation was also assessed. Even in the presence of PTX, signalling was evident in response to MCP-1, -2, -3 ad 4, albeit reduced. This selective inhibition of ERK infers that PTX does not uncouple CCR2 from all classes of G protein.

The effects of PTX on functional response were clear from chemotaxis assays (Figure 5.11). Treatment of THP-1 cells with 100ng/ml PTX totally abrogated all chemotaxis towards MCP-1, confirming earlier observations that CCR2 is linked to the $G\alpha_i$ family of G proteins (Kuang *et al*, 1996; Turner *et al*, 1998).

The role of MAPK in CCR2-mediated chemotaxis

To test the relative importance of ERK in transducing the chemotactic signal of MCP-1, it was necessary to test the effects of MEK inhibitor, PD98059. The compound, PD98059 (Alessi *et al*, 1995), which specifically inhibits MEK1, has been reported to be a potent inhibitor of cytokine-induced ERK-1/2 activation (Alessi *et* al, 1995). The observation that ERK-1/2 is activated by MCP-1, a potent physiological activator of monocytes, does not prove its functional significance in monocyte migration. We endeavoured to evaluate the potential role of ERK in MCP-1-mediated chemotaxis of THP-1 cells. The effect of MAPK inhibition on MCP-1-mediated chemotaxis was examined by incubating cells with/without various concentrations of the MEK inhibitor PD98059 (Figure 5.12). Strikingly, addition of PD98059 did not block MCP-1-induced chemotaxis at any concentration tested (0-50μM), in fact, pre-treatment with this compound served to potentiate cell migration. Certainly in the case of MCP-1, these results suggest that ERK-1/2 activation *per* se does not appear to be necessary for chemotaxis in this system.

To ensure that PD98059 was an appropriate compound to use in this system, we assessed its biochemical effect on ERK-1/2. This revealed a potent inhibition of MCP-1, -2, -3 and -4-induced ERK activation (Figure 5.13). This was a key experiment to determine that a) MCP-induced activation of ERK was a MEK-driven event, and b) to verify that PD98059 was efficient on its target when applied to intact cells in subsequent functional assays.

The contribution of PKC to CCR2-mediated MAPK activation and functional responses.

The observation of PKC as a lipid- and Ca^{2+}-dependent serine/threonine kinase that serves as a cellular receptor for tumour-promoting phorbol ester has implicated PKC in the activation and mitogenesis of a variety immune cells. This has been further substantiated by the demonstration that cellular depletion of PKC by prolonged treatment with phorbol esters, or PKC inhibitory drugs, significantly inhibits cytokine-induced activation (Altman *et al*, 1990; Li *et al,* 2000). Recent years have seen a surge of interest aimed at elucidating the role of PKC in MAPK activation (Takeda *et al*, 2000). In the context of this study, we have attempted to clarify the role of PKC in the coupling of CCR2 to the MAPK pathway and chemotactic responses.

The PKC inhibitor, Ro-320432, served to increase basal levels of phosphorylation of ERK-1/2 in THP-1 cells, but had no effect on MCP-2 ad MCP-4-induced signalling (Figure 5.14). Although the rise in basal phosphorylation may have masked the magnitude to which the ligands induced a biochemical response, MCP-1 and MCP-3 demonstrated appeared to induce a moderate increase in ERK-1/2 activity above baseline. In the case of MCP-1-induced SAPK activation, inhibition of PKC with Ro-32o432 modestly reduced p38 phosphorylation, but completely ablated MCP-1-stimulated JNK1/2 activation (Figure 5.15).

Figure 5.16A. Using a concentration of MCP-1 known to elicit a chemotactic response (1nM), cell were pre-treated with Ro-320432 (0-50μM) and assayed for chemotaxis. Migration was not inhibited by this compound, but was potentiated (although ot significantly) in a concentration-dependent manner. At 50μM Ro-320432, the chemotactic index toward MCP-1 was increase from 2.4 to 2.8. To confirm this data, the effect of PKC on MCP-1-induced cell migration was assessed by depletion of intracellular PKC through long term exposure to phorbol ester (PMA) (Figure 5.16B). In conflict to the Ro-320432 data, pre-treatment of cells with PMA completely abrogated cell migration.

Results Summary

- MCP-1 stimulates activation of ERK-1/2, p38 and JNK-1/2 in a PI3-kinase-dependent manner. Incubation of cells with MCP-1 gave rise to a modest increase in active GTP-bound Ras, as assessed by GST-RBD fusion protein assay.

- ERK-1/2 activation was severely abrogated by LT-82, but only partially reduced by LT-9048. LT-82 inhibited MCP-1-induced PtdIns(3,4,5)P_3 generation and PKB phosphorylation. This toxin also abrogated MCP-1-stimulated *In vitro* lipid kinase activity in p110γ immunoprecipitates.

- LT-82 and LT-9048 attenuated cell migration towards MCP-1.

- MCP-1-stimulated activation of ERK-1/2 and JNK-1/2 were partially inhibited by pertussis toxin, whereas the chemotactic response was completely abolished.

- MEK inhibitor, PD98059, was found to inhibit all CCR2-mediated ERK-1/2 activation, although served to potentiate cell migration towards MCP-1.

- Selective inhibition of PKC isoenzymes by Ro-320432 1) had no visible effect on MCP-1-stimulated ERK-1/2 phosphorylation, 2) a partial inhibitory effect on p38 activity and; 3) totally inhibited JNK-1/2 activation.
- Ro-320432 modestly potentiated MCP-1-induced chemotaxis in a concentration-dependent manner. However, depletion of intracellular PKC stores by long-term exposure to phorbol ester abolished cell migration towards MCP-1.

- MCP-2 stimulated ERK-1/2, p38 and JNK-1/2 in a manner that was partially dependent on PI3-kinase whereas in response to MCP-3, these enzymes were entirely PI3-kinase dependent. On the other hand, when stimulated with MCP-4, ERK-1/2 activation was sensitive to PI3-kinase inhibition, but SAPKs, p38 ad JNK-1/2 were unaffected.

- Pertussis toxin totally inhibited MCP-2, -3, -4 -induced ERK-1/2 phosphorylation, but only partially reduced JNK-1/2 activation.

Figure 5.1 The Effect of wortmannin on CCR2-mediated ERK-1/2 activation in THP-1 cells

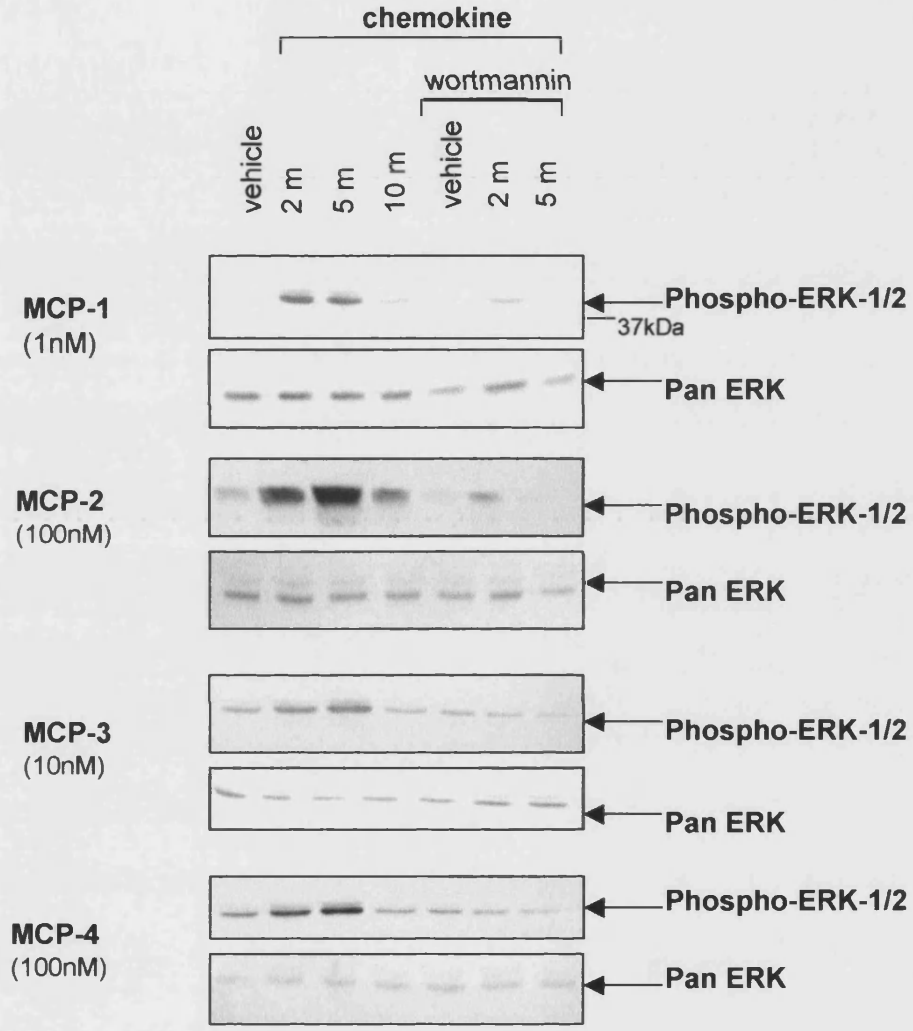


Figure 5.1 The Effect of wortmannin on CCR2-mediated ERK-1/2 activation in THP-1 cells. THP-1 cells (1 x10^6 per point) were pre-treated for 15 minutes with vehicle (0.01% DMSO) or 50nM wortmannin. Cells were then stimulated with vehicle (0.05% BSA), MCP-1, -2, -3 or -4 for the times indicated. Total cell lysates (2.5x10^5 cell equivalents per lane) were resolved separately by SDS-PAGE, electrophoretically transferred to a nitrocellulose membrane, and immunoblotted with anti-active MAPK antibodies (phospho-ERK-1/2). To verify equal loading of proteins, a set of corresponding lysates were probed with anti-pan ERK1 antibody. The results are representative of at least three independent experiments.

Figure 5.2 The Effect of wortmannin on CCR2-mediated SAPK activation in THP-1 cells

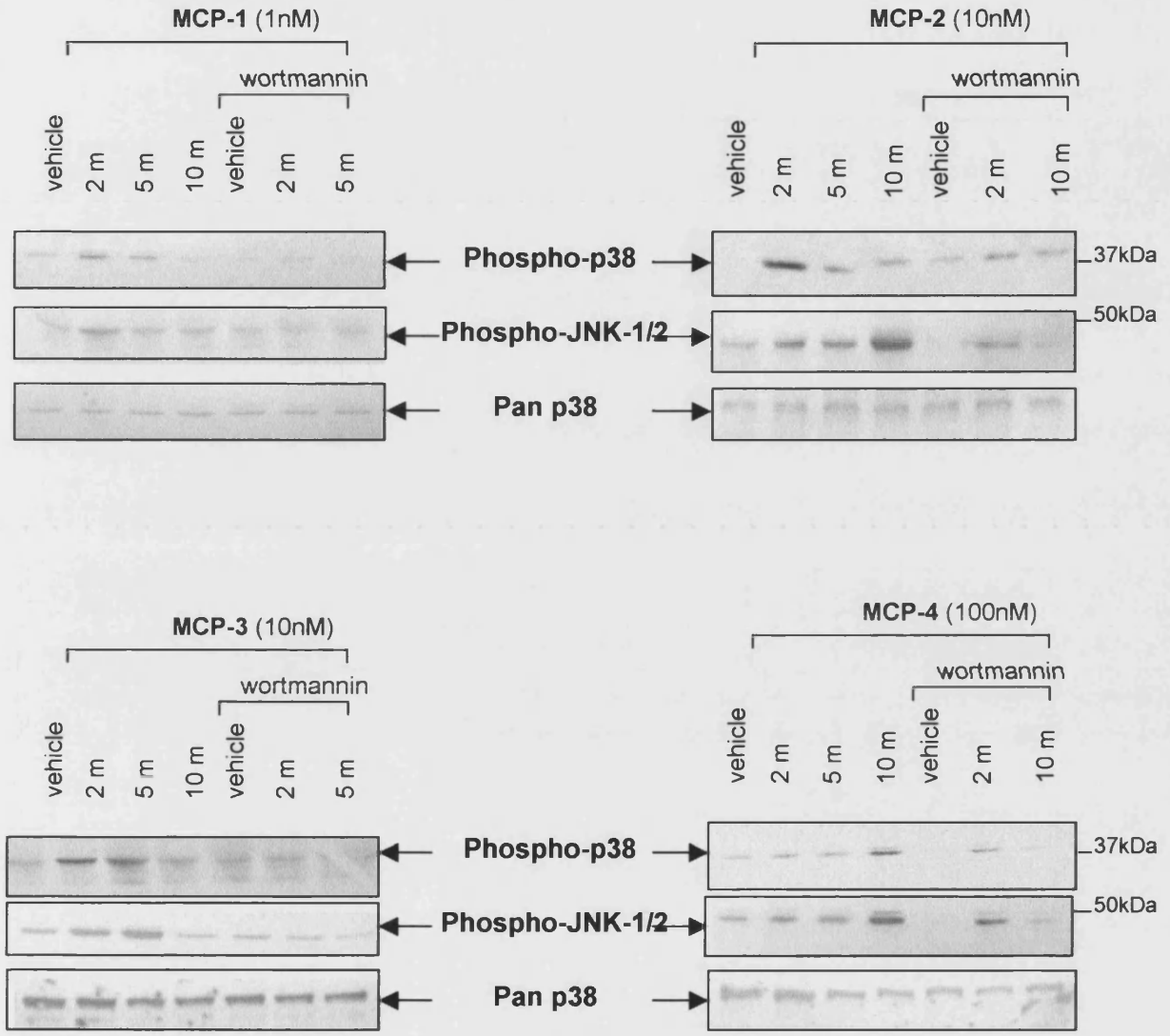


Figure 5.2. The Effect of wortmannin on CCR2-induced SAPK activation in THP-1 cells. THP-1 cells ($1x10^6$ cells/point) were pre-incubated for 15 minutes with vehicle (0.01% DMSO) or 50nM wortmannin. Cells were then stimulated with vehicle (0.05% BSA), MCP-1 MCP-2, -3 or MCP-4 for the times indicated. Total cell lysates ($2.5x10^5$ cell equivalents per lane) were resolved separately on SDS-PAGE, electrophoretically transferred onto nitrocellulose membrane, and immunoblotted with anti-active SAPK antibodies (phospho-p38 or phospho-JNK-1/2). To verify equal loading of proteins, a set of corresponding lysates were probed with anti-pan p38. The results are representative of at least two other experiments.

Figure 5.3 The Effects of MCP-1 on the activation of Ras in THP-1 cells

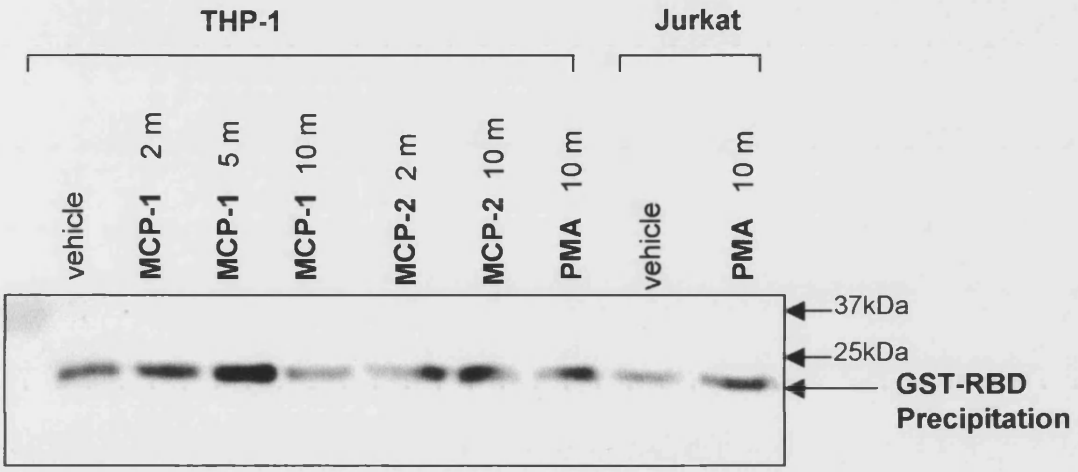


Figure 5.3 The Effects of MCP-1 on the activation of Ras, in THP-1 cells. The plasmid encoding for the GST-RBD fusion protein (provided by Dr D Cantrell) was expressed in bacteria and used to obtain recombinant GST-RBD fusion protein. 1 x10^7 THP-1 cells were stimulated with MCP-1 (10nM) and MCP-2 (100nM) for the times indicated. Cells were lysed, and lysates incubated with GST-RBD fusion protein and glutathione-sepharose beads as described in 'Materials and Methods'. After washing, beads were analysed by 15% SDS-PAGE followed by western blotting with anti-pan Ras antibody. Data is from one experiment and is representative of at least one other.

Figure 5.4 The Effects of *Clostridium sordelli* lethal toxin on ERK-1/2 activation in THP-1 cells

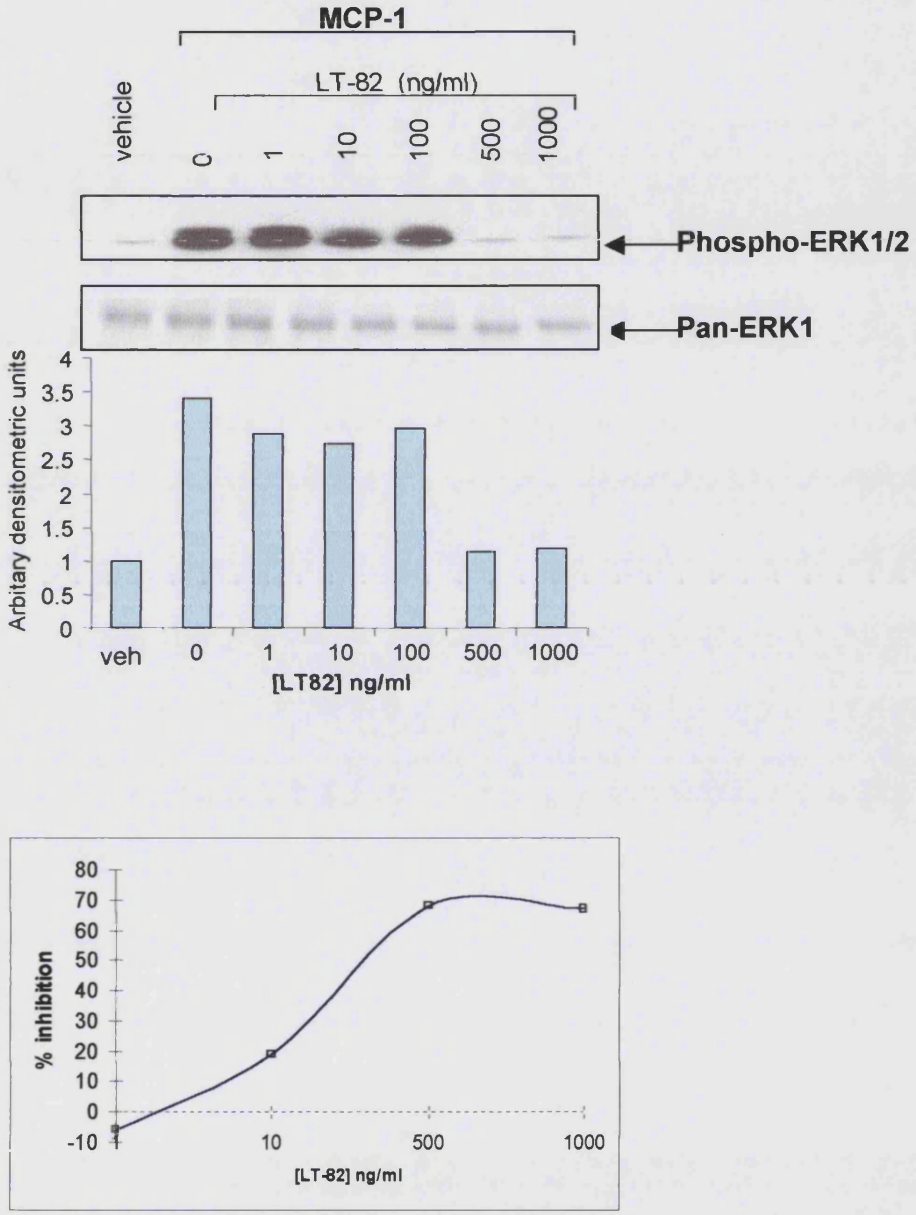


Figure 5.4 The Effects of *Clostridium sordelli* lethal toxin on ERK-1/2 activation in THP-1 cells. 1x10^6 THP-1 cells/point were pre-treated for 2 hours with either vehicle (0.02% DMSO) or various concentrations of LT-82 at 37°C. Total cell lysates (2.5x10^5 cell equivalents per lane) were resolved separately on SDS-PAGE and electrophoretically transferred onto nitrocellulose membrane. Membranes were then immunoblotted with either anti-phospho-ERK1/2 or pan ERK. This figure shows the scanned western blot image along side densitometric analysis expressing results as activity as a % of the control. The results are representative of at least three separate experiments.

Figure 5.5 The Effects of *Clostridium sordelli* lethal toxin on MCP-1-induced ERK-1/2 activation in THP-1 cells.

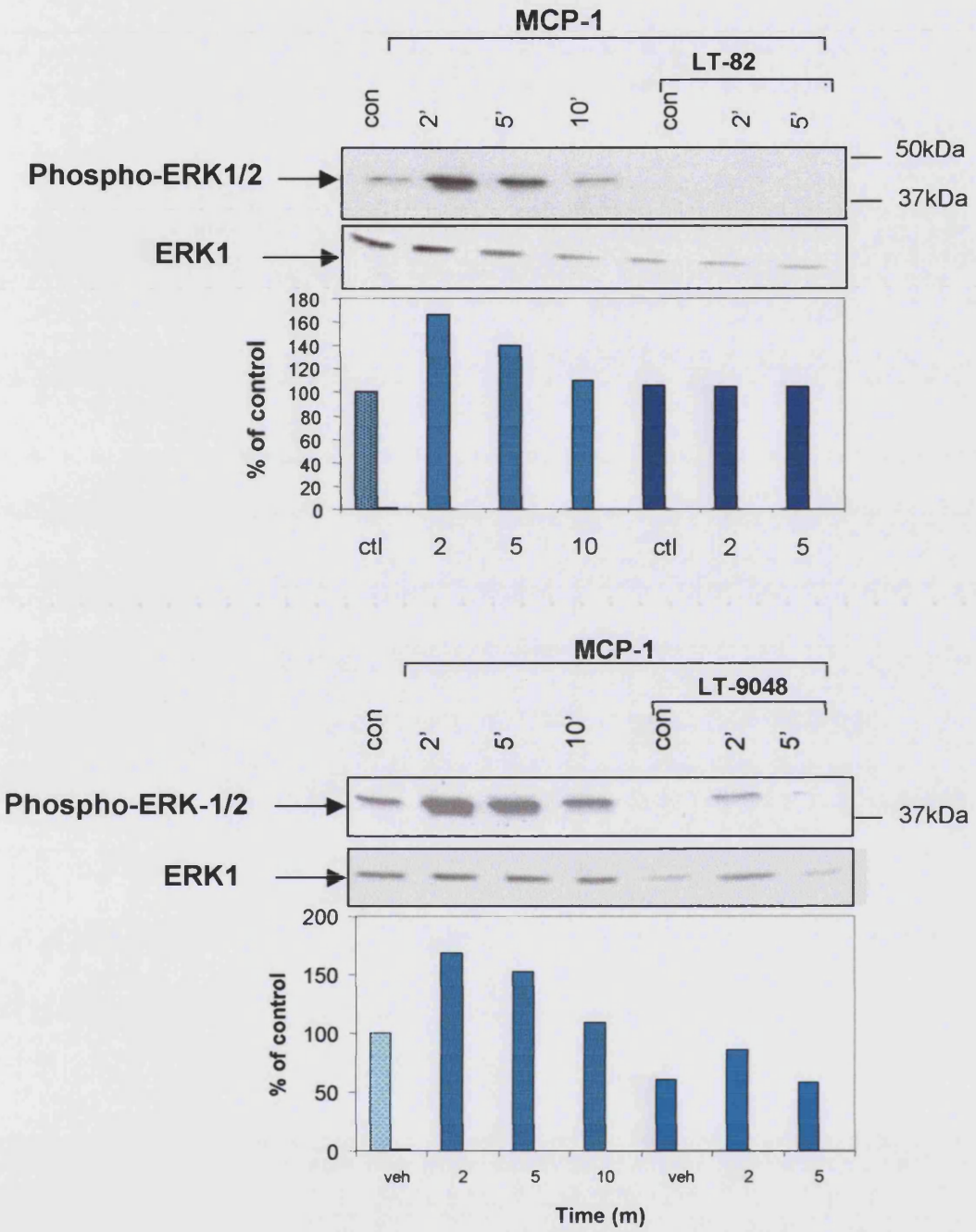


Figure 5.5. The effect of *Clostridium sordelli* lethal toxins on MCP-1-induced ERK-1/2 activation in THP-1 cells. 1x10^6 THP-1 cells/point were pre-treated for 2 hours with either vehicle (0.01% DMSO), LT-82, or LT-9048 (500ng/ml) at 37°C. Total cell lysates (2.5x10^5 cell equivalents per lane) were resolved separately on SDS-PAGE, electrophoretically transferred onto nitrocellulose membrane and immunoblotted with either anti-phospho-ERK1/2, -phospho-PKB, or pan ERK1 antibodies (see also Fig.5.7). This figure shows the scanned western blot image along side densitometric analysis expressing results as activity as a % of the control. Results are normalised the against equal loading blot and are representative of at least three separate experiments.

Figure 5.6 The Effects of *Clostridium sordelli* lethal toxin on MCP-1-Induced PtdIns(3,4,5)P_3 accumulation in THP-1 cells

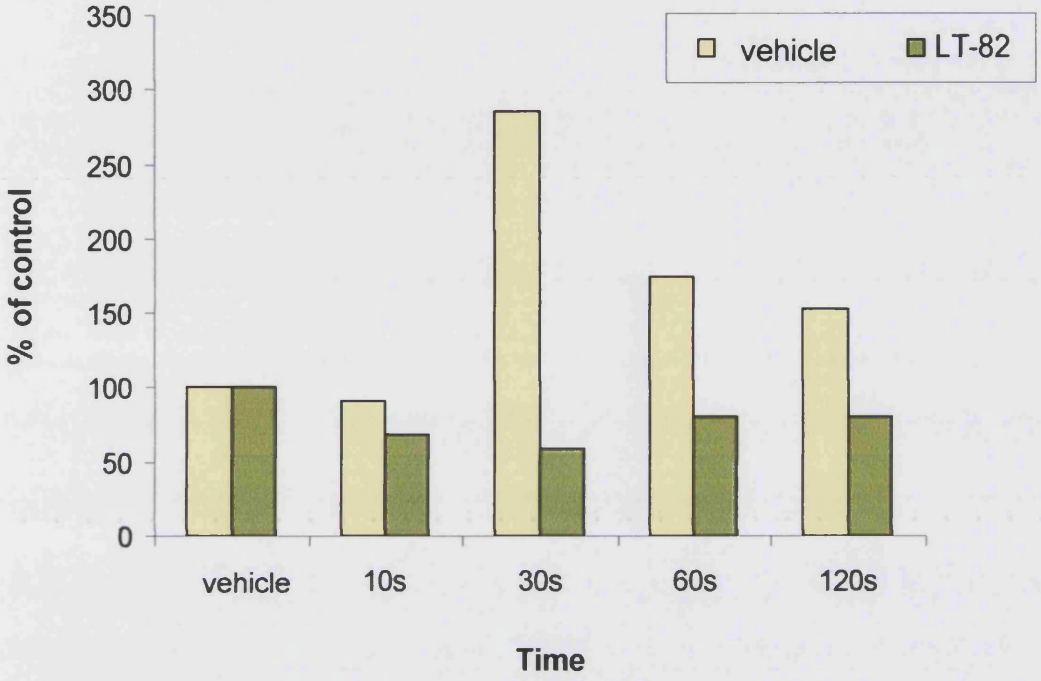


Figure 5.6. The Effect of *Clostridium sordelli* lethal toxin on MCP-1-induced PtdIns(3,4,5)P_3 generation in THP-1 cells. 1 x 10^7 cells/point were metabolically labelled with ^{32}P, and then pre-treated for 2 h with either vehicle (0.01% DMSO) or LT-82. (500ng/ml). Cells were then stimulated with vehicle (0.05% BSA) or MCP-1 (1nM) for the indicated times. Following stimulation, phospholipids were extracted, deacylated and the glycerophosphorylinositol derivatives of PtdIns(3,4,5)P_3 were analysed using HPLC as described under "Materials and Methods". Data are from a single experiment and are representative of at least three separate experiments.

Figure 5.7 The Effect of *Clostridium sordelli* lethal toxin on MCP-1-induced PKB activation in THP-1 cells

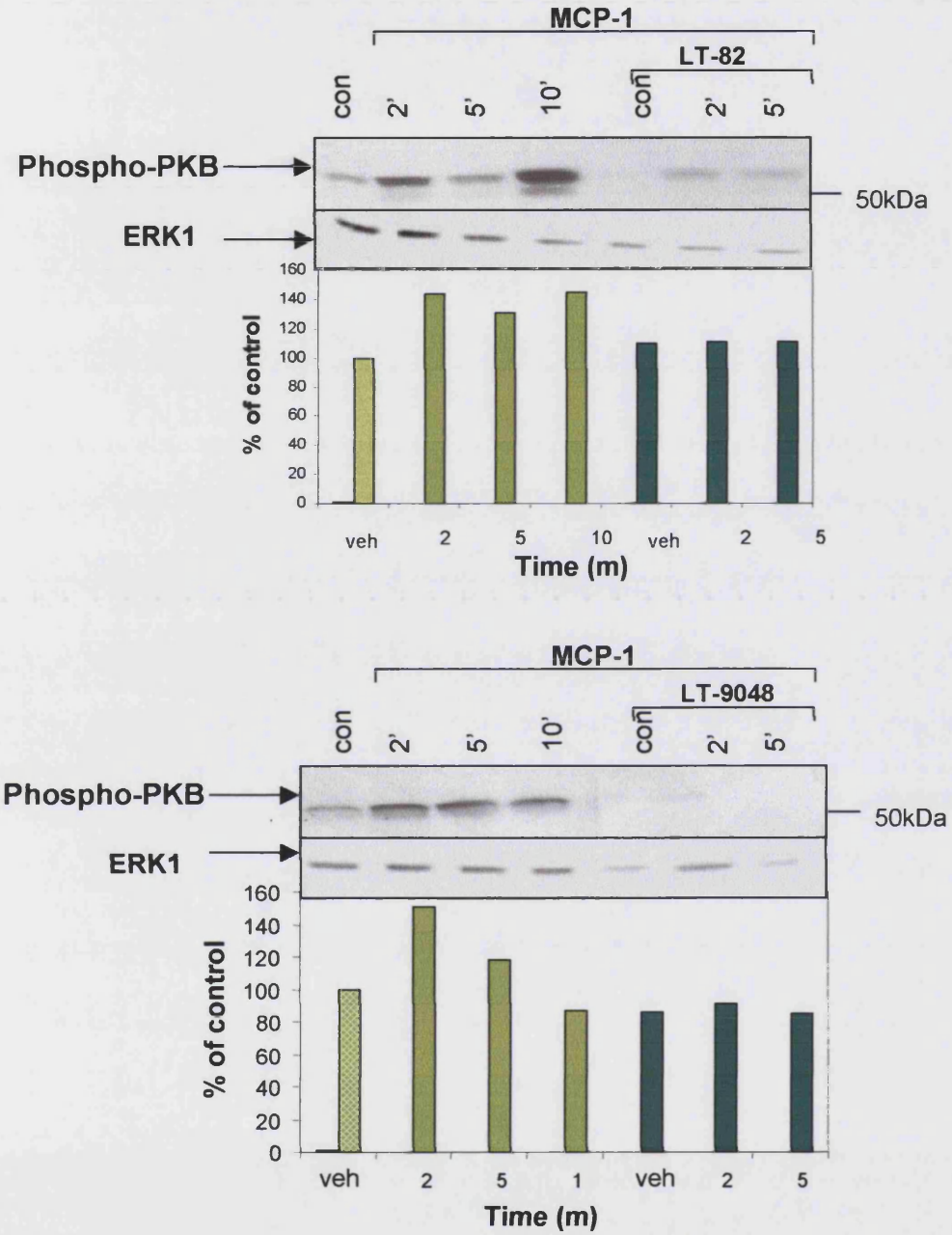


Figure 5.7. The Effect of *Clostridium sordelli* lethal toxin on MCP-1-induced PKB activation in THP-1 cells. 1x10^6 THP-1 cells/point were pre-treated for 2 hours with either vehicle (0.01% DMSO), LT-82, or LT-9048 (500ng/ml) at 37°C. Total cell lysates (2.5x10^5 cell equivalents per lane) were resolved separately on SDS-PAGE, electrophoretically transferred onto nitrocellulose membrane and immunoblotted with either anti-phospho-ERK1/2, -phospho-PKB, or pan ERK1 antibodies (see also Fig. 5.5). This figure shows the scanned western blot image along side densitometric analysis expressing results as activity as a % of the control. Results are normalised against equal loading blot and are representative of at least three separate experiments.

Figure 5.8 Effects of *Clostridium sordelli* lethal toxin on MCP-1-stimulated *in vitro* lipid kinase activity in THP-1 cells.

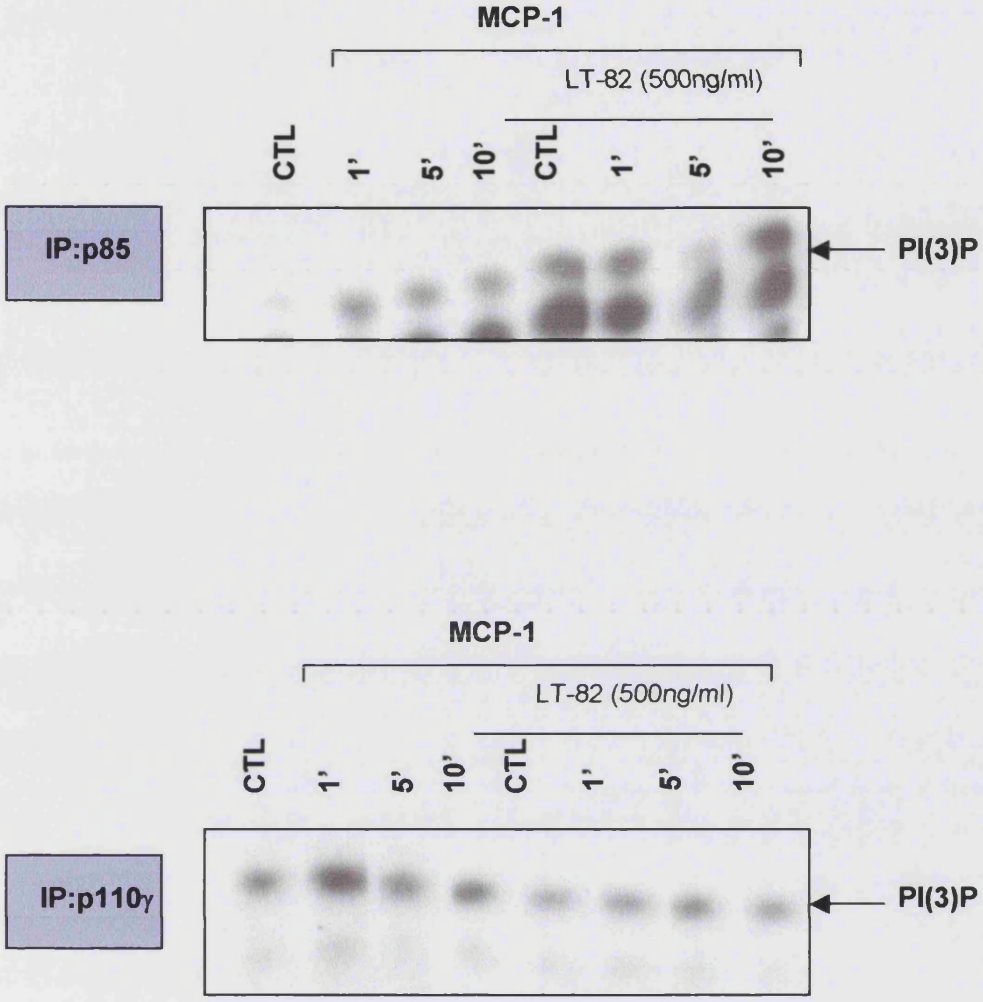
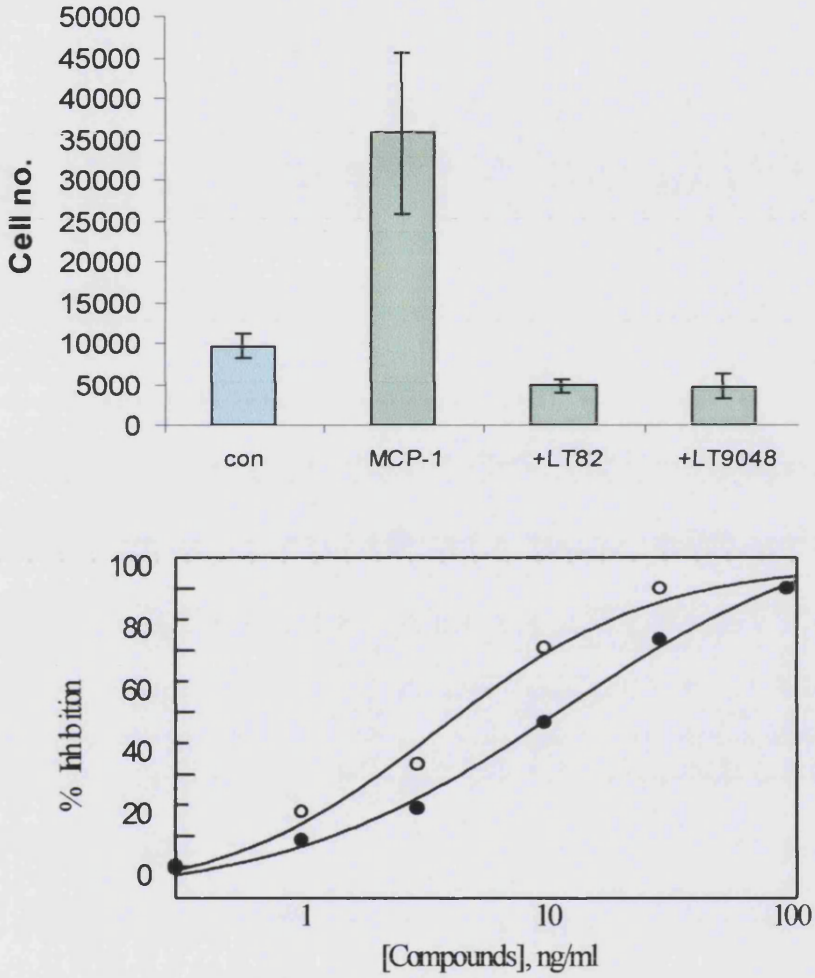


Figure 5.8. Effects of Clostridium sordelli lethal toxin on MCP-1-stimulated *in vitro* lipid kinase activity in THP-1 cells. THP-1 cells ($1x10^7$ per point) were stimulated with MCP-1 (10nM) for the times indicated. Cells were then lysed, and lysates were subjected to immunoprecipitation with either anti-p85 or anti-p110γ mAbs. The washed immunoprecipiates were analysed for PI3K activity using PI as a substrate. Extraction and TLC separation of the lipid products were performed as described under 'Materials and Methods'. Lipids were detected by exposure to film at -70°C. Results are from one experiment and are representative of three separate experiments.

Figure 5.9 The Effect of *Clostridium sordelli* Lethal toxins on MCP-1-Induced Chemotaxis of THP-1 cells



LT-82, IC_{50} = 3.6 ± 0.8 ng/ml	**LT9048** IC_{50} = 11.8 ± 3.4ng/ml

Figure 5.9 Effect of *Clostridium sordelli* lethal toxin on MCP-1 -induced chemotaxis of THP-1 cells. 1.2 x 10^5 THP-1 cells per point were pre-treated for 15 minutes at 37°C with either vehicle (0.01% DMSO) or various concentrations of wortmannin. After incubation, cells were subjected to chemotaxis towards MCP-1 (1nM) in a 96-well Neuroprobe™ chamber for 3 hours. Migration was determined as described in 'Materials and Methods'. Results are expressed as a Chemotactic Index (C:I): the ratio of stimulated over basal migration. Data represent the mean ± S.EM (5 replicates per experiment). Results are representative of three independent experiments (one experiment conducted by K. Moores, Glaxo-SmithKline Pharmaceuticals. Harlow. UK).

Figure 5.10. The Effect of pertussis toxin on MCP-1-induced MAPK activation in THP-1 cells.

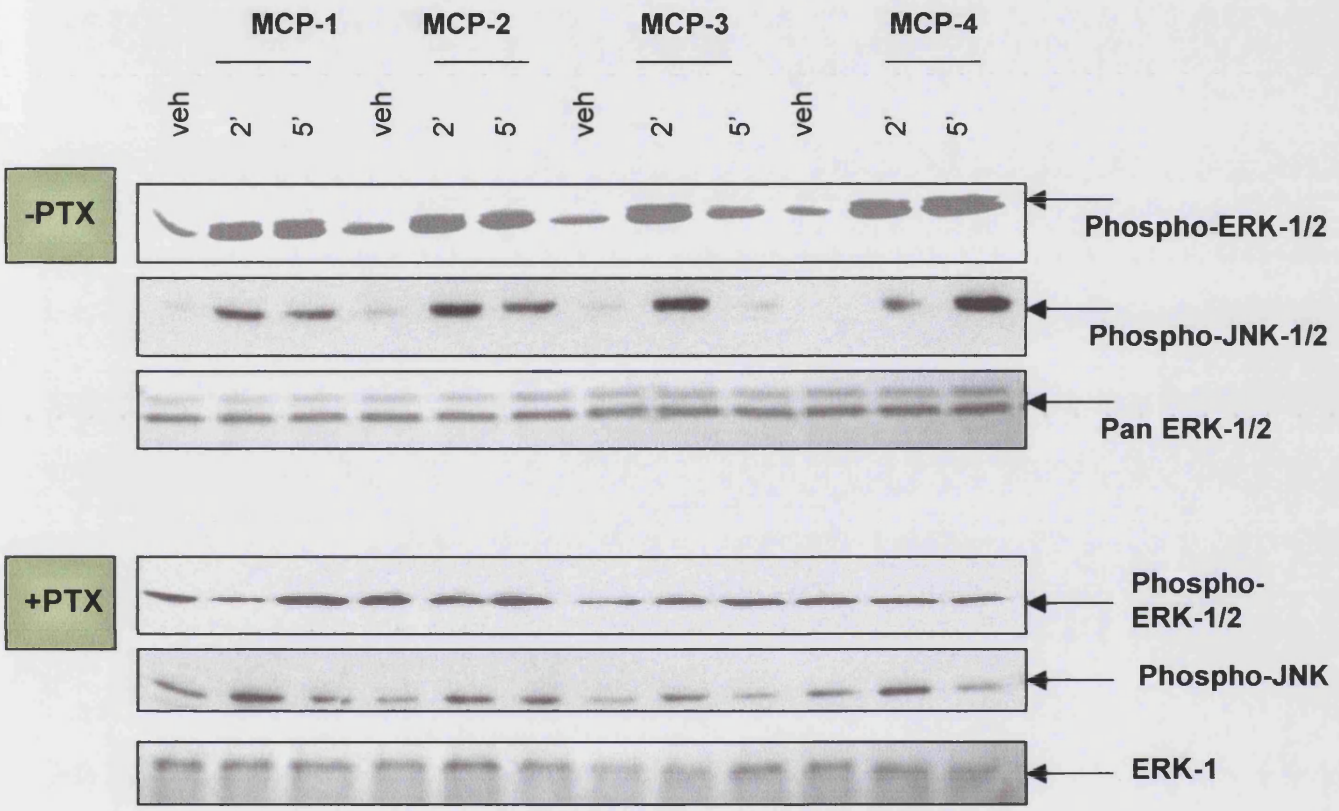


Figure 5.10. The Effect of pertussis toxin on MCP-1-induced MAPK activation in THP-1 cells. THP-1 cells ($1x10^6$ per point) were pre-treated at 37°C for 16 hours with either vehicle (0.01% DMSO) or 100ng/ml PTX. Cells were then stimulated with vehicle (0.05% BSA) or MCP-1, -2, -3, -4 (1, 10, 10, 100nM, respectively) for the times indicated. Total cell lysates ($2.5x10^5$ cell equivalents per lane) were resolved separately on SDS-PAGE, electrophoretically transferred onto nitrocellulose membrane and immunoblotted with anti-phospho-ERK-1/2. To verify equal loading of proteins, a set of corresponding lysates were probed with anti-pan ERK-1/2/ERK-1 antibodies. The results are representative of two experiments.

Figure 5.11 Effect of Pertussis toxin on MCP-1-induced chemotaxis

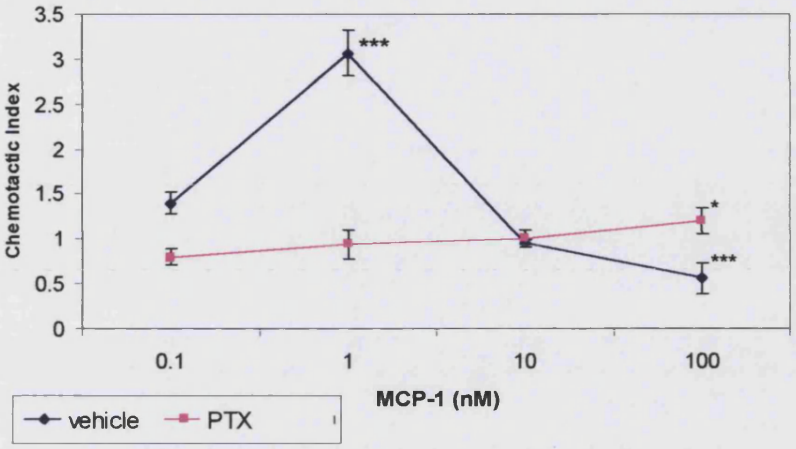


Figure 5.11. Effect of pertussis toxin on MCP-1-induced chemotaxis of THP-1 cells. 1 x 10^5 THP-1 cells per point were pre-treated for 16 hours with either vehicle (0.01% DMSO) or 100ng/ml of pertussis toxin at 37°C. After incubation, cells were subjected to chemotaxis towards various concentrations of MCP-1 in a 96-well Neuroprobe™ chamber for 3 hours. Migration was determined as described in 'Materials and Methods'. Results are expressed as a Chemotactic Index (C:I): the ratio of stimulated over basal migration. Data represent the mean ± S.EM (5 replicates per chamber/2 chambers per experiment). Results are representative of four independent experiments. *: p <0.05, ** : p<0.01, *** : p<0.001

Figure 5.12 The Effect of MEK inhibitor, PD98059, on MCP-1-induced chemotaxis of THP-1 cells

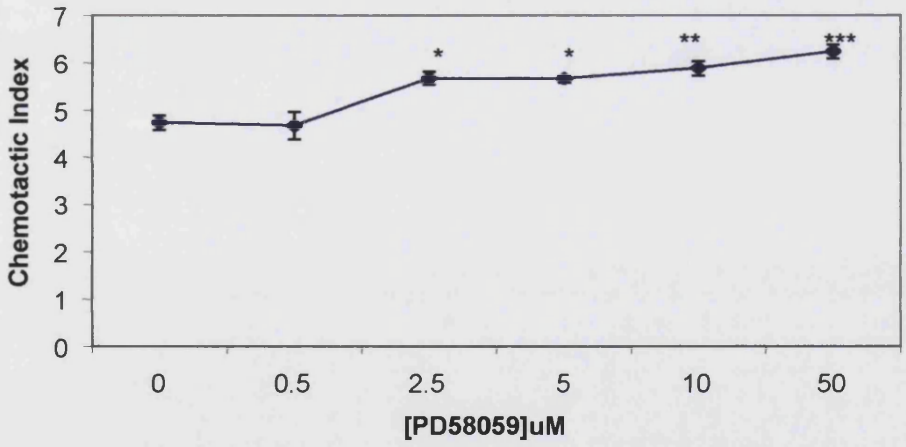


Figure 5.12 Effect of PD98059 on MCP-1-induced chemotaxis of THP-1 cells. 1 x 10^5 THP-1 cells per point were pre-treated for 30 minutes with either vehicle (0.01% DMSO) or indicated concentrations of PD98059 at 37°C. After incubation, cells were subjected to chemotaxis in a 96-well Neuroprobe™ chamber for 3 hours. Migration was determined as described in 'Materials and Methods'. The concentration of MCP-1 in the lower chamber was 1nM - a concentration determined by previous dose-response experiments (Section 4). Results are expressed as a Chemotactic Index (C:I): the ratio of stimulated over basal migration. Data represent the mean ± S.EM (5 replicates per chamber/2 chambers per experiment) Results are representative of four independent experiments. *: p <0.05, ** : p<0.01, *** : p<0.001

Figure 5.13 Confirmation of ERK phosphorylation as being a MEK-driven event.

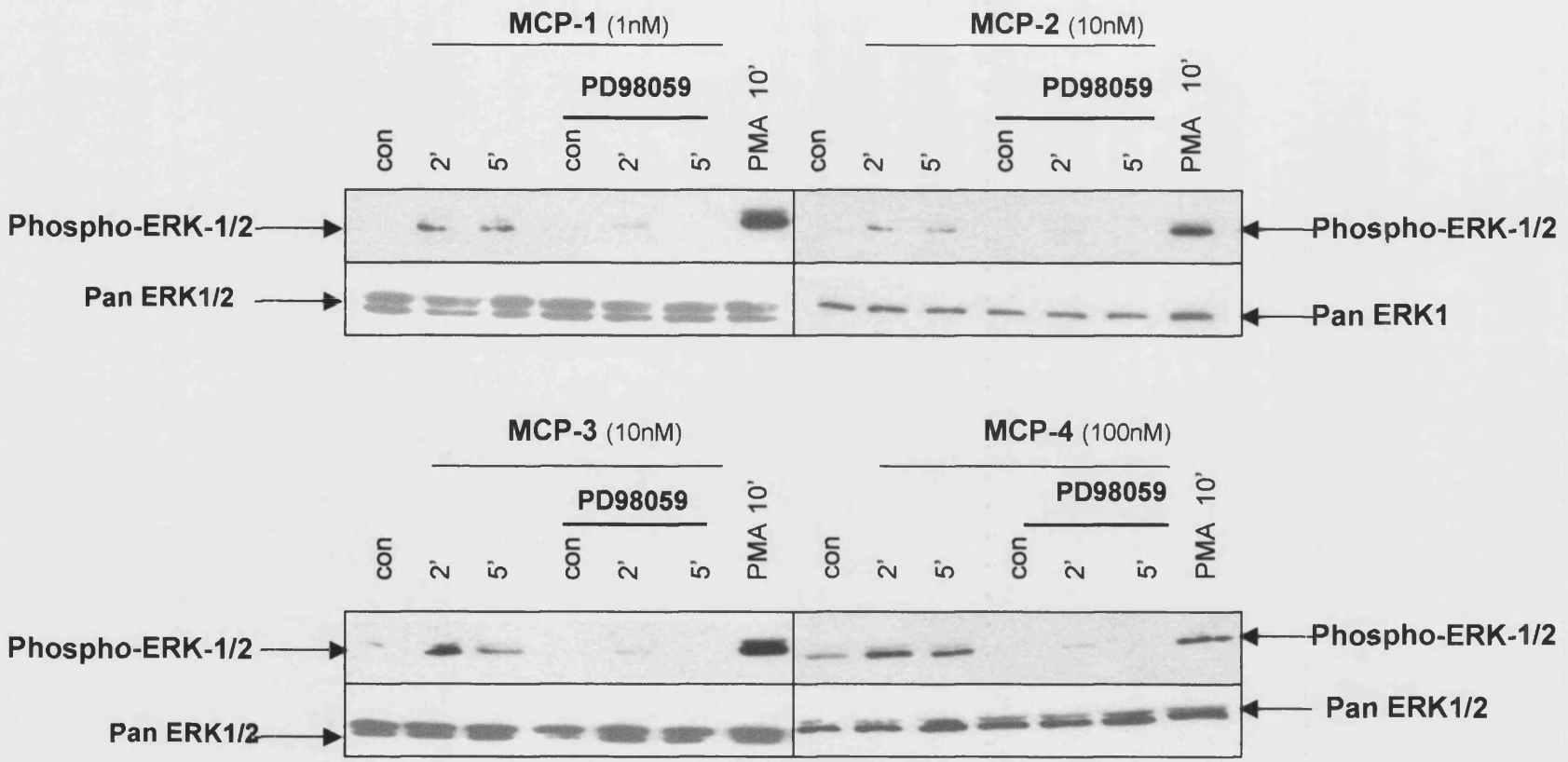


Figure 5.13 Confirmation of ERK phosphorylation as being a MEK-driven event. THP-1 cells ($1x10^6$/cells per point), untreated or pre-incubated for 15 minutes with vehicle (0.01% DMSO) or 10μM PD98059. Cells were then stimulated with vehicle (0.05% BSA), PMA (5ng/ml^{-1}) or MCP-1-4 for the times indicated. Total cell lysates ($2.5x10^5$ cell equivalents per lane) were resolved separately on SDS-PAGE and electrophoretically transferred onto nitrocellulose membrane. Membranes were then immunoblotted with anti-phospho-ERK-1/2 antibody. Membranes were subsequently stripped (as described in 'Materials and Methods') and re-probed with an anti-ERK-1/anti-ERK-1/2 antibody, to ensure equal loading of proteins. The results are representative of two experiments.

Figure 5.14 The Effect of PKC inhibitor, Ro-320432, on ERK-1/2 activation in THP-1 cells.

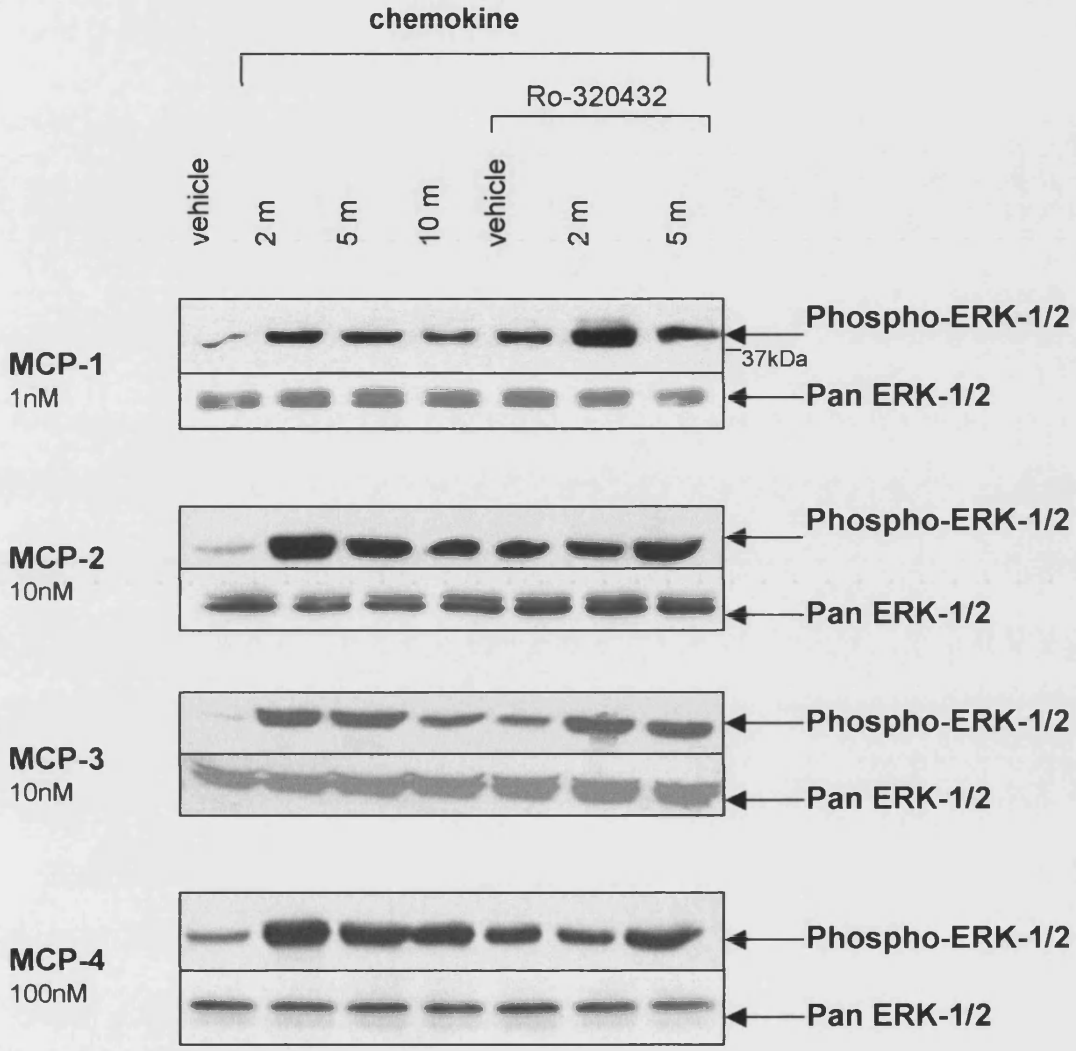


Figure 5.14 The Effect of PKC inhibitor, Ro-320432, on ERK-1/2 activation in THP-1 cells. THP-1 cells ($1x10^6$ per point) were pre-treated at 37°C for 1 hour with either vehicle (0.01% DMSO) or 10μM Ro-320432. Cells were then stimulated with vehicle (0.05% BSA), MCP-1, MCP-2, MCP-3 or MCP-4 for the times indicated. Total cell lysates (cell equivalents of $2.5x10^5$ per lane) were resolved separately by SDS-PAGE, electrophoretically transferred to a nitrocellulose membrane, and immunoblotted with anti-phospho-ERK-1/2 antibody. To ensure equal loading of proteins, another set of corresponding lysates were probed with pan-ERK1/2 antibody. The remaining lysates were stored at -70°C for further investigation. The results are representative of three independent experiments.

Figure 5.15 The Effect of PKC inhibitor, Ro-320432, on MCP-1-induced SAPK activation in THP-1 cells

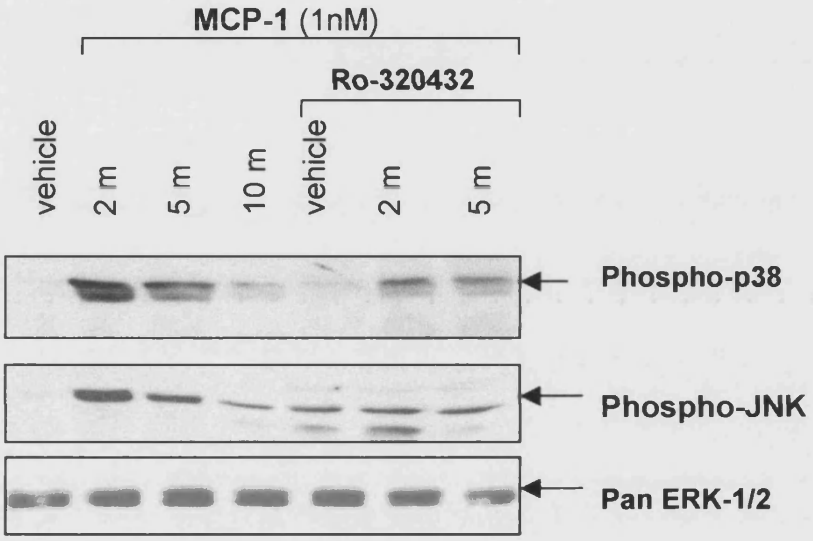
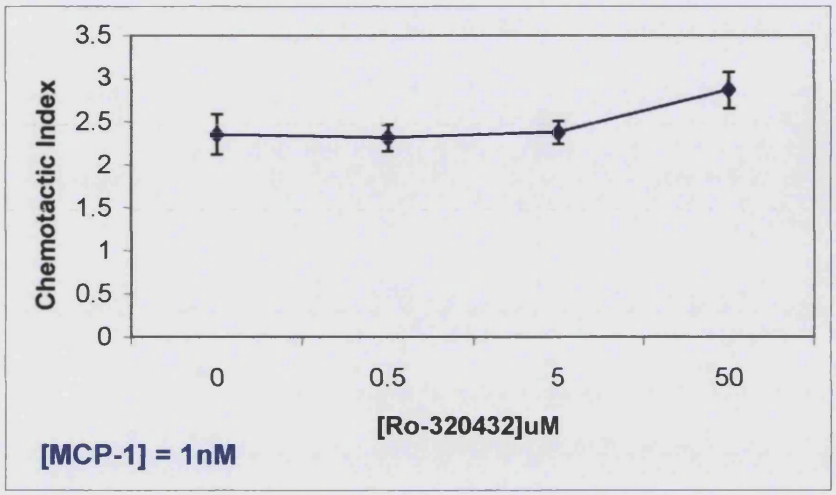


Figure 5.15 The Effect of PKC inhibitor, Ro-32-0432 on MCP-1-induced SAPK activation in THP-1 cells. THP-1 cells ($1x10^6$ per point), were pre-treated at 37°C for 1 hour with either vehicle (0.01% DMSO) or 10μM Ro-320432. Cells were then stimulated with vehicle (0.05% BSA) or MCP-1 (1nM) for the times indicated. Total cell lysates (cell equivalents of $2.5x10^5$ per lane) were resolved separately by SDS-PAGE, electrophoretically transferred to a nitrocellulose membrane and immunoblotted with anti-active SAPK antibodies (phospho-p38 and phospho-JNK-1/2). To ensure equal loading of proteins, a set of corresponding lysate were immunoblotted with anti-pan ERK-1/2. The results are representative of three experiments.

Figure 5.16 The Role of PKC in MCP-1-mediated chemotaxis

A)



B)

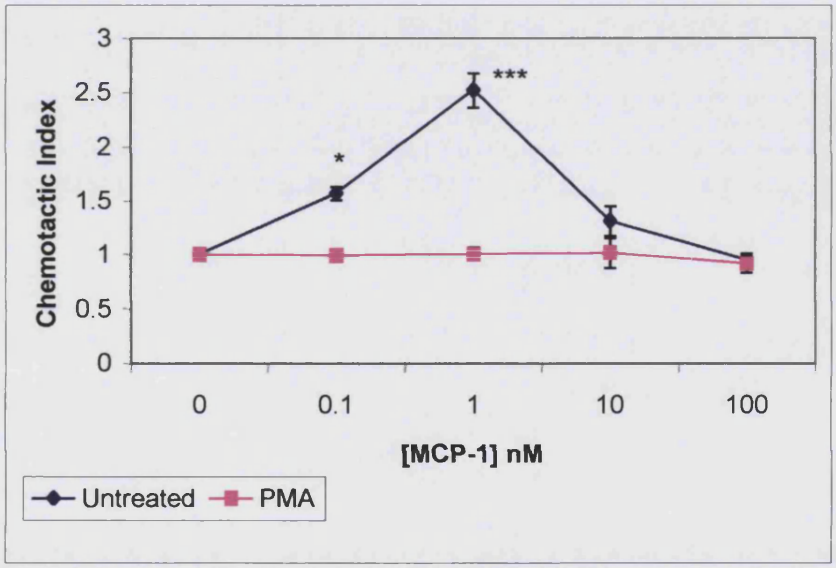


Figure 5.16 The role of PKC in MCP-1-mediated chemotaxis (A) 1 x 10^5 THP-1 cells per point were pre-treated with either vehicle (0.01% DMSO) or with the indicated concentrations of Ro-320432 for 30 minutes at 37°C. **(B)**. 1 x 10^5 THP-1 cells per point were pre-treated either vehicle (0.01% DMSO) or 100ng/ml^{-1} PMA for 16 hours at 37°C. After incubation, cells were subjected to chemotaxis in a 96-well Neuroprobe™ chamber for 3 hours towards indicated concentrations of MCP-1. Migration was determined as described in "Materials and Methods". Results are expressed as a Chemotactic Index (C:I) : the ratio of stimulated over basal migration. Data represent the mean ± SEM (5 replicates per chamber/2 chambers per experiment). Results are from one experiment, but are representative of three others. *: $p < 0.05$, ** : $p < 0.01$, *** : $p < 0.001$

Section 5. Discussion

The Role of PI3-kinase in MAPK activation.

The question of how GPCRs control signals that regulate the gene expression in the nucleus, even though intensively studied during the last few years, is not yet fully answered. It has been shown that GPCRs can activate MAP kinases, ERK1/2, c-Jun N-terminal kinase and p38 MAPK and they represent a point of convergence for cell surface signals regulating cell growth and division (Marshall *et al*, 1995). Several studies have now shown that CXC (e.g. SDF-1 and IL-8) as well as CC chemokines (eotaxin, MIP-3α, MCP-1) stimulate the phosphorylation of MEK-1 and or ERK-1/2 in a variety of systems (Knall *et al*, 1997; Sullivan *et al*, 1999; Sotsios *et al*, 1999). Although significant heterogeneity exists between receptors and cells types, the main theme in this research is to elucidate the involvement of certain signalling intermediates linking these receptors to the MAPK/SAPK cascade.

1) Coupling of CCR2 to the MAPK cascade.

We present here that MCP-2, -3 and –4 signals via CCR2 and activate ERK in a PTX-sensitive manner, and can therefore conclude that CCR2 is a $G\alpha_i$-coupled receptor. MCP-1-stimulated activation of ERK-1/2 appears to be only partially sensitive to PTX, and therefore suggests that there is a degree of heterogeneity in the coupling of CCR2 to G-proteins in the ERK-1/2 pathway. It is widely accepted that both pertussis toxin (PTX)-sensitive and -insensitive heterotrimeric G-proteins mediate ERK activation, and distinct signalling pathways are ascribed to GTP-bound α and free βγ G-protein subunits. $G\alpha_i$-coupled receptors preferentially utilise a G-βγ route via PI3K-γ, which feeds into the "classical" MAPK pathway involving Ras (Crespo *et al,* 1994; Lopez-Ilasaca *et al*, 1997).

However, Gβγ subunits derived from PTX-insensitive $G\alpha_q$ proteins have also been implicated in Ras-dependent ERK activation. For instance, $G\alpha_q$-coupled receptors may employ PKC to target Raf-1 directly or calcium to activate MAPK via Pyk2, Src and Ras (Della Rocca *et* al, 1997; Dikic and Blaukat, 1999). Although G_i-coupled receptors are unanimously viewed as coupling to ERKs in a Ras-dependent manner, previous studies have produced contradictory results as to Ras-dependent or -independent ERK activation via G_q-coupled receptors in COS cells, and there are

several studies providing evidence of chemokine receptors coupling to G_q-proteins (Wu *et al*, 1993). Furthermore, dominant-negative forms of Ras do not inhibit ERK-1/2 activation in IL-8-stimulated CHO transfectants, which again suggests that chemokines can mediate Ras-independent ERK-1/2 activation (Shyamala *et al*, 1998). It is becoming increasing evident in many systems that this Ras-independent ERK-1/2 activation may potentially involve PI3Kγ activation.
It must be highlighted that not all GPCRs mediate MAPK activation exclusively via the receptor catalysed release of G-$\beta\gamma$ subunits. In 1995, van Biesen *et al* reported that activation of ERK-1/2 in COS-7 cells was mediated by the α-subunit of a PTX-sensitive G_0-protein that was independent of Ras activation.

Delineating the precise mechanisms by which CCR2 couples to the ERK-1/2 pathway is complicated by the diversity of possible signalling mechanisms and heterogeneity exhibited by chemokine receptor signalling. We shall now schematically consider the constituent signalling events arising from CCR2 engagement that culminates in ERK-1/2 activation, and attempt to elucidate whether these biochemical responses assume functional relevance in this system.

2) The Role of Small GTP-binding proteins in MCP-1-Induced MAPK activation

The use of a fusion proteins consisting of a GST-tagged Ras binding domain has demonstrated that MCP-1-stimulation of THP-1 cells is associated with increased activation of Ras. On the whole, there is very little information directly linking Ras activation with chemokine stimulation, although our group has previously reported that SDF-1 activates Ras in Jurkat cells, and suggested further biochemical and functional roles for this GTPase (Sotsios *et al*, 1999). In these studies, however, the broad-spectrum farnesyl-transferase inhibitors were used as a method of assessment (e.g. Manumycin), and so Ras activity could only be inferred in this system. IL-8-stimulated activation of Ras and Raf-1 has also been reported in neutrophils with the use of transfected isoforms of Ras; Here, K-Ras4B was significantly more effective in mediating Raf-1 migration than H-Ras, K-Ras4A and N-Ras (Voice *et al*, 1999). More recently, Ras activity has been detected in CXCR3 stimulated Hepatic stellate cells (HSC), and was shown to be a key player in PI3K and MAPK pathways (Bonnachi *et al*, 2001). In terms of Ras activation and its involvement in CCR2-mediated signal transduction, MCP-1 was the only ligand analysed against the receptor during this study, and showed a modest increase in Ras activity in THP-1. The relevance of Ras and other small GTPases in MAPK

activation and other cellular responses was further analysed using selective bacterial toxins.

Lethal toxin (LT) from *Clostridium sordelli* is a glucosyltransferase that covalently modifies 21kDa proteins *in vitro* and *in vivo*, and glucosylates Ras, and to a lesser extent Rap, Rac, Ral and Cdc42 (Popoff *et al*, 1996). This is the first toxin to inactivate Ras, and is becoming an invaluable tool to explore pathways regulated by GTPases. Here, we have shown that LT completely abrogates ERK1/2 phosphorylation in response to MCP-1, and conclude that Ras, at least in part, functions upstream of the MAPK cascade in this system. We also conducted experiments to analyse the effect of other toxins on MCP-1-induced chemotaxis. LT, as expected, completely abrogated MCP-1-stimulated chemotaxis of THP-1 cells. Toxin B (from *Clostridium difficile*) that inactivates Rho, Rac and Cdc42 (Just *et al*, 1995), also blocked migration towards MCP-1. Although we did not continue further investigation with Toxin B, this data suggests that these proteins were instrumental in chemotaxis and may have been implicated at a number of points in the pathway. For example, Rho GTPases could be acting upstream of SAPKs which themselves could be regulating cell migration. It is also possible that Toxin B abrogates the GTPase-regulation of Phospholipase D (PLD), a strong candidate in the control the cytoskeletal dynamics involved in cell migration. In short, Rho GTPases are likely to play a regulatory role wherever filamentous actin is used to drive a cellular process, namely cell shape, polarity, morphogenesis and chemotaxis.

Although LT would block Rap and Rac as well as Ras, the inhibiting effect on Ras would predominate, because of order of selectivity of this toxin. Therefore, in our system we have assumed that the observed effects were due to Ras inhibition alone. However, we cannot categorically rule out that Rac and Ral are also components of this pathway since these GTPases function upstream and downstream of Ras, respectively. The possible involvement of Rac in MCP-1-mediated ERK-1/2 activation is highlighted by the fact that LT-82, but not LT-9048 completely inhibited the ERK-1/2 pathway (LT-9048 does not modify Rac proteins). It is also conceivable that Rap may be involved in this cascade considering that recent reports have pointed to Rap as a positive regulator in cell signalling, however, its major role has been considered to be as an antagonist towards Ras (Boussiotis *et* al, 1997). The use of constitutively active or dominant negative mutants of various G proteins, along side the clostridial toxins could have yielded more definitive results of G protein

involvement in MCP-1 action. However, application of these highly specific toxins has paved the way for future investigation.

Ras: Upstream or downstream of PI3K?

Before we go on to discuss the role of PI3K in CCR2-mediated MAPK activation, it is necessary to consider the current issues facing the relationship between Ras and PI3K. Ras exerts its effects through a GTP-dependent interaction with several cellular targets including class I PI3Ks, and all class I PI3Ks have a Ras binding domain (RBD) similar to those found in other effectors such as Raf and Ral (Vanhaesebroeck *et al*, 2001). Although there is clear evidence of the interplay between these signalling molecules, it is emerging that PI3K is not a universal effector as once thought.

In our system, treatment with LT clearly abolished the MCP-1-stimulated generation of PI3K lipid products as assessed by D-3-lipid accumulation and severely reduced PKB phosphorylation. Examination of individual PI3K isoforms also revealed that p110γ-associated *in* vitro lipid kinase activity was inhibited by treatment with LT. The effects of LT on the class II PI3K-C2 were not assessed, although it would have been useful given that this isoform is considered to be main contributor of the overall D-3 phosphatidylinositol lipid pool. Although we have shown here that Ras lies upstream of PI3K in this system – whether or not it has a dual function downstream of PI3K remains to be determined.

3) PI 3-kinase – A key player in MCP-Induced ERK activation?

Several studies have now indicated that PI3K inhibitors can abrogate chemokine-induced ERK activation. This correlates well with our findings in that phosphorylation of ERK-1/2 in response to MCP-1, -2, -3 and –4-stimulation was significantly inhibited with wortmannin. Although we have shown in numerous studies that ERK-1/2 is phosphorylated by these chemokines, we found there to be a high degree of variability between basal levels of MAPK between experiments, which appeared to be exacerbated by washing and spinning down of cells. Such variability in THP-1 cells has been previously observed, and in support of our theory, MacKenna *et al*, reported that in fibroblasts, ERK and JNK were prematurely activated by 'mechanical stretch'. Much effort was made to minimise the effects of cellular stress on biochemical and functional studies.

It is interesting to note that in COS cells, LPA-induced activation of Ras and MAPK is blocked by inhibitors PI3K or by dominant-negative inhibitors of the p85 subunit of PI3K. This is a strong indication that that p110α or p110β isoenzymes are required for MAPK activation at a point upstream of Ras (Hawes *et al*, 1996). The PI3Kγ isoenzyme has also been shown to act upstream of Ras in the G-βγ subunit activation of ERK-1/2 in COS cells. In this respect, activation of MAP kinases via G-βγ subunit complex mediated pathway is dependent upon tyrosine phosphorylation, Ras activation, and distal protein phosphorylation cascades (Ablas *et al*, 1993; Faure *et al*, 1994). This hypothesis was later refined and it was shown that the MAPK cascade is activated by the intrinsic protein kinase activity of PI3Kγ, which represent a point of bifurcation from its lipid kinase activity (Hirsh *et al*, 2000). Further evidence for the role of G-βγ subunits and p110γ in MAPK activation was gleaned from $p110\gamma^{-/-}$ knockout mice. In this study, it was demonstrated that $p110\text{-}\gamma^{-/-}$ knockout mice displayed a significantly reduced activation of ERK (Li *et al*, 2000).

If we consider MCP-1 stimulation of CCR2, our experiments suggest that ERK-1/2 activation is $G\alpha_i$-coupled receptor event that appears to be dependent on PI3K, and Ras, but not PKC. It could follow that MCP-1 induces the "classical" growth factor signalling cascade that involves Shc, Grb2, Ras, Raf. Since we did not investigate the contribution of Ras to MCP-2, -3 or –4-induced ERK activation, we cannot speculate on the dependency of this GTP binding protein to the MAPK cascade in response to these ligands. However, it can be concluded that all four chemokines activate ERK1/2 via a $G\alpha_i$-coupled receptor, and that PI3K activity is a prerequisite in each case. Preliminary experiments have shown that it is only in response to MCP-2 and MCP-4 that dual inhibition of PI3K and PKC attenuates activation of ERK-1/2 (data not shown). Here, it is conceivable that PI3Kγ may involve the action of PKCζ, which has been shown to occur via a PDK-1-dependent manner as shown in Figure 5.17 (Chou *et al*, 1998).

More recently, Sonnenburg *et al*, have shown that PDK-1 can phosphorylate and activate conventional PKC isoenzymes independently of PI3K. This could present an alternative mechanism whereby PKCζ is allosterically controlled by PDK-1 independently of the PH domain or 3-phosphoinositides. In neutrophillic models, PKCξ is also thought to play multiple roles leukocyte transmigration (Laudanna *et al*, 1998). The biological consequences of such diversity in chemokine receptor signalling is not totally understood – although it may offer some explanation as to

how chemokines exert control a over a multitude of functional and transcriptional responses.

4) Stress Activated Protein Kinases (SAPKs) in CCR2-mediated signalling

In a similar fashion to the activation of the ERK pathway by proliferative stimuli, UV radiation, environmental stress and proinflammatory cytokines stimulate the JNK/SAPK and the P38 MAP kinase (p38 MAPK) cascades. These kinases have been shown to activate a number of transcription factors, including c-Jun and ATF-2 (Kyriakis *et al*, 1994). We present evidence here that in the THP-1 cells line, MCP-1, -2, -3 and -4 induced activation of the SAPKs, namely p38 and JNK.

5) The c-Jun N-terminal kinase pathway – JNK/SAPK

We investigated the effects of MCP-1, -2, 3 and –4 on the activation of the JNK signalling pathways. All of which induced a rapid and transient phosphorylation of this enzyme. Previously, only a few studies have shown chemokine receptors to activate the JNK pathway, including CCR2 and the Herpesvirus-8 receptor, ORF74 (Pati *et al*, 2001). In our system, the PI3K inhibitor, wortmannin, served to completely abrogate JNK-1/2 activity in response to MCP-1 and MCP-3, but was less efficient in the inhibiting JNK-1/2 in response to MCP-2 and MCP-4. This is an indication that CCR2 is able to couple to the JNK-1/2 via more than one pathway.. Several inflammatory cytokines, such as TNFα and EGF have been reported to activate JNK in a PI3K-depedent manner (Kim *et* al, 1999; Logan *et al*, 1997), although this has not yet been shown in the context of chemokine signalling. Cambien *et al* demonstrated that JNK activation by MCP-1 was a $G\alpha_i$-mediated event dependent protein tyrosine kinase activity. Interestingly, among the best-characterised JNK activators that act downstream of tyrosine kinases are members of the PI3K family.

With respect to GPCRs coupling to the PI3K-dependent JNK pathway, the role of P3K-γ in G$\beta\gamma$-dependent regulation of JNK has been well characterised. What is more interesting, is that signalling through this mechanism suppressed by dominant negative mutants of Ras, Cdc42 and Rac (Lopez-Ilasaca *et al*, 1998). Giving further evidence that PI3K serves to regulate the small GTP-binding proteins upstream of SAPKs that may ultimately facilitate cell migration. The expression of mutants in monocytic cells has indicated that Cdc42 regulates rearrangement of the

cytoskeleton in response to MCP-1 and MIP-α. MCP-1 and MIP-1α, but not Cdc42-stimulated cytoskeletal reorganisation can be inhibited by wortmannin. An indication that PI3K is involved at a point upstream of Cdc42 in CC chemokine-stimulated migration (Weber *et al*, 1998). It would have been interesting to assess the role of small GTPases, such as Ras and Cdc42, in CCR2-mediated activation of JNK-1/2 using the clostridial lethal toxins.

The observation that PKC, as well as PI3K, may function upstream of JNK-1/2 leads us to propose an alternative mechanism whereby phosphoinositides produced by PI3K stimulate PKCζ activation via PDK-1, and then subsequent generated second messengers then proceed to activate JNK. This is based on the observation that a) phosphoinositide-regulated kinase, PDK-1, can activate PKC; b) our data infer that PDK-1 is operational in this system (See Section 5), and c) PKCζ has been previously shown to activate ERK-1/2 in a PI3K-dependent manner. Procyk et al have proposed a similar mechanism in LPS-stimulated macrophages. Here, the JNK pathway is activated by a PI3K-dependent, Cdc42/Rac-independent mechanism involving PKCζ and the generation of ceramide as a second messenger.

Indirect interaction of PKC-ζ with JNK has also been observed in other systems and is thought to form complexes with MEKK1, SEK and JNK/SAPK in myeloid cells. In terms of functional relevance, several other PKC isoenzymes have been identified as regulators of JNK activation subsequent monocytic differentiation, and in the pathogenesis of myeloid leukaemia (Kaneki *et al,* 1999). In the CCR2-induced activation of JNK observed in our system, the involvement of PI3K probably occurs at a point upstream of the four 'tiers' of protein kinases, and participates in the cascade soon after engagement of the receptor. This quite possibly involves interaction with small GTP-binding proteins such as Cdc42, Rac or Ras. Alternatively, the PI3K could be sequentially activating PKC through PDK-1 modulation, and activating JNK in a manner independent of small GTP-binding proteins.

6) Activation of the p38/MAPK pathway via CCR2

Like JNK, the p38 MAPK pathway has not been as extensively studies as the prototypical ERK/MAPK pathway, particularly in terms of chemokine activation. However, recent reports have suggested p38 to be a pivotal kinase involved in chemotaxis induced by serum, lysophosphatidyl-choline, and chemokines in smooth muscle cells and leukocytes (Jing *et al*, 2000). MCP-1 has previously been shown to

activate SAPKs (Yamasaki *et al*, 2001), and in this study, we not only reiterate these findings but also show that MCP-2, -3 and -4 may also activate these pathways. Cambien *et* al, recently reported SAPKs, but not ERKs to be implicated in MCP-1-induced MonoMac6 cell migration. These data, along with other recent investigations corroborate with our findings that ERK is clearly not involved in THP-1 cell CCR2-mediated chemotaxis in THP-1 cells (Ashida *et al*, 2001; Fine *et al,* 2001). Figure 5.18. The upregulation of chemokine proteins themselves are thought to be dependent upon p38, e.g. the production of MCP-1 in endothelial cells and IL-8 in neutrophils (Waterhouse *et al*, 2001; Marie *et al*, 2001). Taken together, these data suggest that activation of p38, and indeed JNK may facilitate the autocrine-like control of cell behaviour by their respective chemokines in inflammatory states.

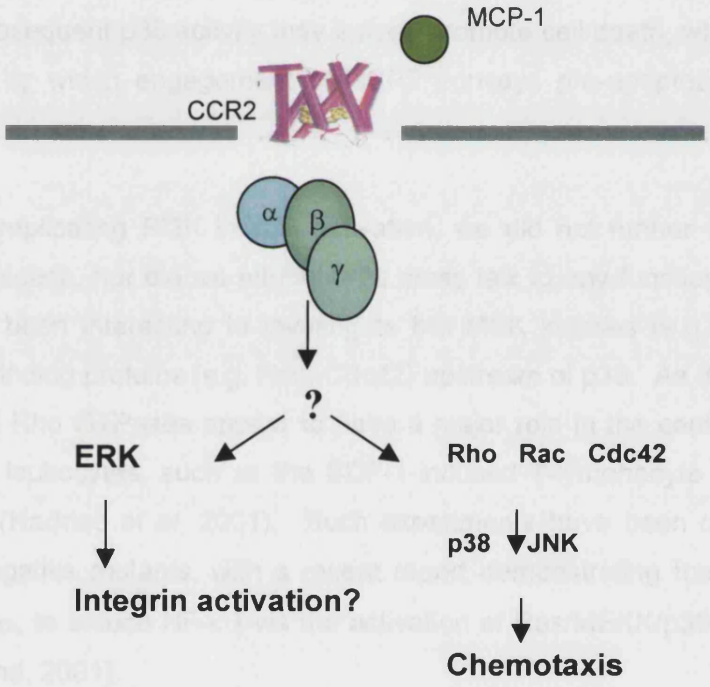


Figure 5.18 A proposed model to explain MCP-1-mediated chemotaxis. MCP-1 activates MAPKs, ERK, p38 and JNK, and stimulates chemotaxis of THP-1 cells. MCP-1-mediated chemotaxis is not dependent on ERK in our system, and we postulate that p38 and/or JNK could be necessary for cell migration. Obviously, we cannot attribute the biological roles of p38 and JNK without the appropriate functional data. However, we can make inferences from our preliminary data and from previous studies by other workers. Ashia *et al* showed that activation of ERK is required for integrin activation, whereas p38 was required for chemotaxis in a Rho-dependent manner. Two biological functions mediated by MCP-1 may utilise two distinct MAPK-dependent signalling pathways.

Inhibition of the PI 3-kinase pathway allowed us to clearly establish that PI3K is involved in the activation of the p38 cascade by CCR2. The concept that there may

be an element of interplay between p38 and the PI3K/PKB is not without precedent. In a recent study by Jing *et al*, p38 and PI3K was shown to functionally associate with and be critically involved in THP-1 cell chemotaxis induced by oxidised-LDL. Cross-talk is also suggested by the PKB-p38 regulation of cytoprotection versus apoptosis in VEGF-stimulated endothelial cells (Gratton *et al*, 2001).
Transcriptional regulation by these pathways has also been extensively studied. The controversial mechanism by which PKB activates NF-κB has been reported to have a dependency on p38 activation, and consistent with this is the requirement of p38 in IL-1β-induced transcriptional control in several systems (Madrid *et al*, 2001). With respect to CCR2-mediated signalling, it would be reasonable to assume that p38 could be an important target of the PI3K/PKB pathway may explain how the CCR2 ligands could exert transcriptional control in this system. Conversely, regulation of PKB and subsequent p38 activity may indeed promote cell death, which could offer a mechanism by which engagement of CCR2 conveys pro-apoptotic signals to the monocyte.

Apart from implicating PI3K in p38 activation, we did not further characterise this signalling cascade, nor did we attribute the cross talk to any functional relevance. It would have been interesting to investigate the MEK kinases (e.g. MEKK1) or the small GTP-binding proteins (e.g. Ras, Cdc42) upstream of p38. As discussed earlier, the Ras and Rho GTPases appear to have a major role in the control of directional migration of leukocytes, such as the SDF-1-induced T-lymphocyte chemotaxis and polarisation (Haddad *et al*, 2001). Such experiments have been carried out using dominant negative mutants, with a recent report demonstrating the CXCR2 ligand, MGSA/GROα, to induce NF-κB via the activation of Ras/MEKK/p38 cascade (Wang and Richmond, 2001).

In the case of MCP-1-stimulated cells, our results suggest a minor role for PKC as a mediator of p38 activation. Here it appears that p38 is downstream and partially dependent upon PKC. This is consistent with reports for the role of PKC-dependent activation of p38 in non-cardiac myocytes. More relevantly, the thrombin-induced signalling pathway exhibits a PKCδ-dependent activation of p38 and this particular interaction has been shown to facilitate ICAM-1 mediated adhesion of leukocytes to endothelial cells (Rahman, 2001). This concept could bear particular relevance in our system in that MCP-1-induced PKC-dependent-activation of p38 could be in place to facilitate the upregulation of integrins subsequent to adhesion of leukocytes

during cell migration. It would be interesting to identify the specific PKC isoforms that appear to be involved in the CCR2-activation of JNK and p38, and perhaps dissect the interactions between each kinase and its respective substrate. However, the use of PKC inhibitors to answer such questions is complicated by the lack of isoenzyme specificity, and that PKC inhibitors may themselves stimulate JNK and p38 activity.

7) CCR2 exhibits differential coupling to PTX-sensitive and –insensitive MAPK pathways.

Studies over recent years have demonstrated that the multitude of proliferative signals elicited by one receptor are likely to be owed to the divergent MAPK cascades involved in connecting GPCRs to the nucleus. Until very recently, GPCRs have been shown to stimulate ERKs through a number of linear pathways that are initiated by either $G\alpha_i$ or $G\alpha_q$ proteins. Previous investigation of CCR2-mediated signal transduction pathways has revealed that MCP-1 activates PLC activation intracellular calcium, inhibition of adenyl cyclase and PI3K (Sozzani *et al*, 1993; Turner *et al*, 1998) in a PTX-dependent manner suggesting a coupling this receptor to G_o/G_i proteins. It has been demonstrated, however, that there can be considerable potential for heterogeneous coupling of chemokine receptor isoforms to different G protein and/or subunits depending on the receptor, the ligand and the cell line studied (Arai and Charo, 1996).

To evaluate the coupling mechanisms of CCR2 to downstream G proteins involved in MAPK activation, THP-1 cells were treated with pertussis toxin. PTX acts to uncouple $G\alpha_{i/o}$ proteins by ADP-ribosylating these subunits at a conserved carboxyl-terminal domain cysteine residue (Jones *et al*, 1987), and is therefore an invaluable tool for studying the function of G_i proteins. We found that ERK-1/2 and JNK-1/2 exhibited differential sensitivity to pertussis toxin (PTX). ERK activation was almost completely abrogated PTX, inferring that this pathway is under the regulation of G_i proteins, whereas JNK was only partially sensitive, and therefore regulated (at least I part) by G_s/G_q proteins. The effect of PTX on p38 activity was not assessed. These data strongly imply that CCR2 exhibits heterogeneity in its coupling to the MAPK pathway via the G protein family.

In several cellular settings, it has been observed that ERK can be potently activated by with G_q-coupled or G_i-coupled receptors, respectively, in a pertussis toxin-

insensitive and –sensitive fashion (Gutkind, 1998). In many cases, both α and $\beta\gamma$ subunits liberated from these G proteins can engage distinct pathways to ERK activation (Ablas *et al*, 1993; Howe *et al*, 1993). Blaukat *et al* have recently shown that dually coupled GPCRs require co-operation of $G\alpha_i$ and $G\alpha_q$-mediated pathways for efficient stimulation of the ERK cascade. Although this may not be the case in our system, this study has highlighted that co-operative signalling by multiple G proteins might represent a novel concept in GPCR-mediated cell regulation.
A more detailed hypothesis of CCR2-mediated ERK activation in THP-1 cells is discussed above. In short, we believe that ERK activity is regulated by a G_i-protein coupled mechanism that induces activation of MEK and ERK1/2 through a pathway that exhibits differential reliance on PI3K and PKC depending on the chemokine studied.

The activation of SAPKs by G protein subunits is thought to occur by a novel biochemical route. Despite the increasing knowledge about the prominent role of JNK in gene transcription, relatively little is known about the signal transduction pathways leading to its activation, particularly by GPCRs. Using the PDGF receptor model in NIH 3T3 cells, activation of GPCRs was shown to induce transcription of early immediate gene, c-jun, through phosphorylation of JNK. Interestingly, activation of GPCRs and not tyrosine kinase receptors for PDGF led to the activation of JNK. Given that this response did not correlate with MAPK activation, it established GPCR signalling pathways diverge at the level of JNK from those utilised by tyrosine kinase receptors (Coso *et al*, 1995). A recent study has also shown that the Kaposi's Sarcoma Herpes Virus-GPCR can be activated by a variety of downstream substrates that lead to the activation of JNK family members, but not MAPK. Proline-rich tyrosine kinase 2 (Pyk2) and Src-family kinase, Lyn, were shown to be pivotal signalling enzymes in this JNK pathway (Munchi *et al*, 1999).

In contrast to the ERK cascade, there is little evidence placing Ras upstream of JNK, instead, it is thought that Rho GTPases Rac1 and Cdc42 initiate an independent kinases cascade regulating JNK activity. Additionally, the Rac/Cdc42 pathway to JNK has been strongly linked to actin polymerisation. So the question is; At what level does the bifurcation of signals to ERK and SAPKs take place? It is quite possible that this may occur at the G protein level, and as inferred from our data, differential coupling to G protein subfamilies can lead to divergence in MAPK activation.

8) Coupling of $G\alpha_i/G\alpha_q$ signals to the chemotactic response

Our functional data complies with previous reports that monocyte migration is a PTX-sensitive process (Sozzani *et al*, 1994) in that THP-1 cell migration toward MCP-1, -2, -3 and –4 was completely ablated by treatment with this toxin. However, this prompted us to investigate relationships that may exist between the MAPK pathways and THP-1 migration towards MCPs 1-4. It is clear that CCR2 not only exhibits heterogeneity in coupling to G protein subunits, but can also link to both PTX-sensitive (G_i) and PTX-insensitive (G_q) family members

Another explanation is in that more than one splice variants and/or receptors are activated by MCP-1, -2, -3, and 4 in this model, and these may differentially couple to different signalling pathways and functional events. Indeed, while MCP-$1^{-/-}$ and $CCR2^{-/-}$ mice have similar phenotypes, they are not similar in other respects (Boring *et al*, 1998; Gosling *et al*, 1999). It is interesting that activation of the MAPKs is under the regulation of distinct signalling mechanisms. The observation that CCR2 can exert independent control of signalling pathways is yet more evidence that chemokine receptors could mediate not only cell movement, but also gene transcription and cell growth.

The dependence on operational G_i proteins for chemotaxis to MCP-1, -2, -3 and –4 corroborated with previous reports. In these studies, inhibition of chemotaxis by PTX was proposed to occur as long as $G\beta\gamma$ release was dependent on $G\alpha_i$ activation. This arose from the observation that neither $G\alpha_i$-GTP nor its effects on cAMP were considered a prerequisite to migration, however, the $G\beta\gamma$ release following $G\alpha_i$ activation was shown to be a key mediator (Arai *et al*, 1997; Neptune *et al*, 1999). A similar mechanism has been reported for IL-8 signalling; Here, the coupling of CXCR2 with various G-protein isoforms was possible, but only G_i, and not G_s or G_q, was necessary for chemotaxis (Neptune *et al*, 1997).

It is unlikely that inhibition of cell migration by PTX occurred due to uncoupling of CCR2 to the ERK-1/2 pathway because studies with MEK inhibitor, PD98058, revealed that ERK-1/2 was not involved in this response. With regards to the precise role of G_i proteins in CCR2-mediated chemotaxis, we can merely speculate. PTX could be exerting its influence on chemotaxis by inhibiting one or more PI3K isoforms, or arresting the recruitment of PLCβ, PKCζ or other downstream signalling

molecules. Alternatively, PTX could be impeding the internalisation and recycling of CCR2 by down-regulating GRK-mediated receptor modification via clathrin-coated pits or recruitment of arrestins. $G\alpha_i$ modification could be reducing overall receptor surface expression and, hence, decreasing the potential for MCP-induced signals for migration. Although, it has been clearly shown in some systems that the events of $G\alpha_i$-coupling chemokine receptors such as CXCR2 and CXCR4 do not have a major role in the regulation of internalisation (Feniger-Barish *et al*, 2000; Sotsios, unpublished work).

9) Involvement of PKC in cell migration

Protein kinase C (PKC) is a major serine-threonine kinase that regulates multiple intracellular events for which 11 isotypes have been reported. The discovery of these various isotypes of PKC and the postulation that they have distinct physiological functions have provided an explanation for the plethora of events, including migration, that appear to be mediated by PKC. An integral role for PKC in cell migration is supported by the observation that; a) many cytoskeletal proteins (e.g. talin, filamin) are substrates for PKC, and; b) downregulation of PKC with phorbol esters results in changes in cell shape that are likely to be an effect of cytoskeletal rearrangement (Mochly-Rosen *et al*, 1990).

The role of PKC activation in cell migration has been not been widely studied, but is thought to be cell specific. Activation of PKC enhances migration of neutrophils, monocytes and endothelial cells (Mercurio *et al*, 1988; Niggli *et al*, 1993), whereas in keratinocytes and some T cells lines, activation of PKC inhibits cell migration (Ando *et al*, 1993). Our initial studies showed that inhibition of PKC with Ro-320432 served to potentiate, rather than inhibit migration of THP-1 cells towards MCP-1. From this it could be postulated that PKC may be exerting inhibitory control of chemotaxis.

If this were the case, what would be the mechanism by which PKC produced this inhibitory effect on chemotaxis? Prior studies have revealed that activators of cAMP inhibit chemotaxis of rat smooth muscle cells (Itoh *et al*, unpublished observations), Moreover, an association between cAMP and PKA signalling pathways has previously been established. Stimulation of human SMCs with forskolin, a potent activator of cAMP, strongly inhibits the PDGF-induced migration of SMCs (Itoh *et al*, 2001). It is therefore plausible that the inhibitory effect of PKC on THP-1 chemotaxis might have been mediated through the cAMP/PKA pathway. To investigate this

hypothesis, it would have been necessary to measure PKA activity is response to Ro-320432 pre-treatment (Figure 5.19).

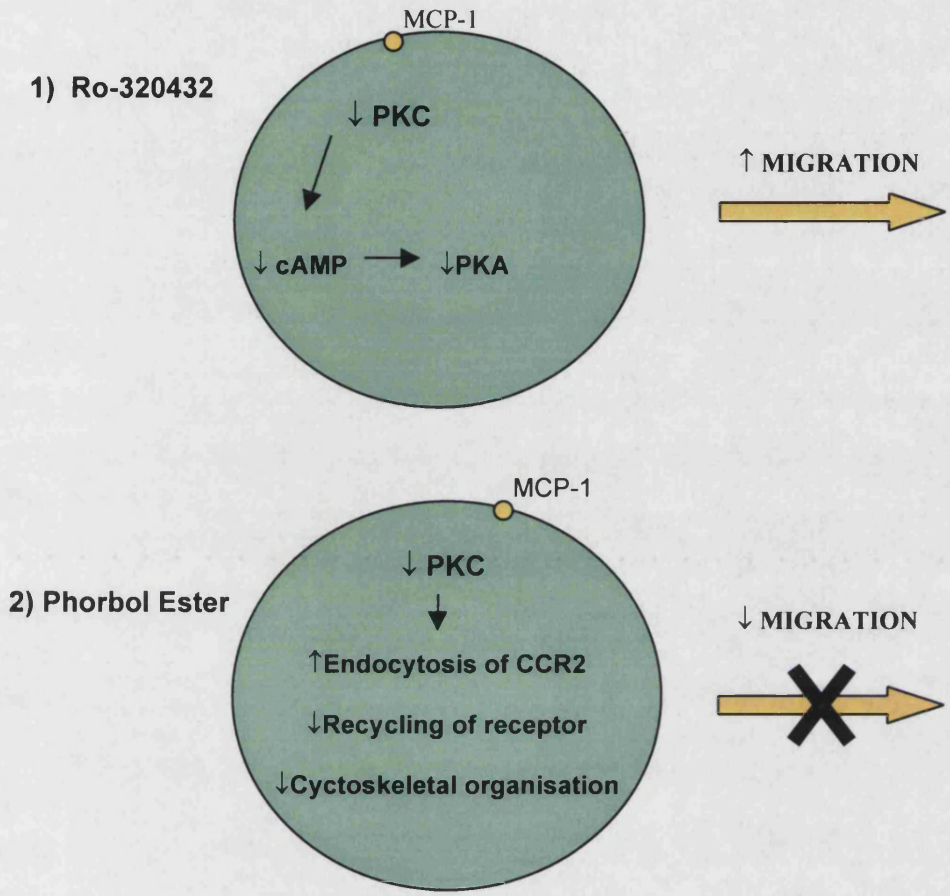


Figure 5.19. Hypothetical model of PKC-involvement in THP-1 cell migration. This diagram offers an explanation of how two different methods of inhibiting PKC could produce conflicting results.

1) Treatment with Ro-320432 potentiates migration. Dowregulation of PKC with Ro-320432 could enhance migration by activating the cAMP/PKA pathway
2) Long-term exposure with phorbol ester totally abrogated chemotaxis. This may be due to PMA-induced effects on CCR2 receptor expression through PKC-mediated endocytosis and/or recycling. Cytoskeletal organisation may also be refractory to PKC modulation by phorbol ester.

To test further test the hypothesis, PKC was subject to inhibition by a second method of downregulation, whereby intracellular PKC isoforms are depleted by prolonged exposure to phorbol ester. Downregulation with PMA did not corroborate with results

obtained with the Ro-320432 inhibitor, as pre-treatment with PMA completely abrogated chemotaxis. The inhibitory action of PMA could have arisen from a number of mechanisms. As well as disrupting PKC-modulated cytoskeletal organisation, phorbol esters could affect migration through downmodulation of CCR2, thus, rendering cells unresponsive to MCP-1 stimulation. This has been shown in the rapid endocytosis of CXCR4 by phorbol ester (Signoret *et al*, 1997). Here, the mechanism of PMA-induced CXCR4 internalisation is thought to involve PKC-mediated phosphorylation of the CXCR4 cytoplasmic domain. Recycling of the receptor back to the membrane could also have been under the control of PKC, and therefore attenuated by phorbol ester treatment. Presented here is rather conflicting data regarding the role of PKC in MCP-1-induced chemotaxis, it is impossible to attribute a definitive role for PKC in this functional response.

SAPKs but not ERKs modulate CCR2-chemotaxis?

Consistent with other reports of chemokine signalling, it appears that ERK-1/2 is not necessary for MCP-1-directed migration of THP-1s (Knall *et al*, 1997). Inhibition of MAPK activation using the specific inhibitor of the kinase responsible for activating MAPK (MAPKK or MEK), PD98059, revealed a potent inhibition of this enzyme, but not the migratory response induced by MCP-1. Although MAPK activation has been reported necessary for MCP-1-induced migration in monocytes, it is so far unclear exactly what role such activation plays because this enzyme can be placed downstream of such effectors as Ras and Rho (both implicated in migration events) as well as PI3-K. The differences in sensitivity to MEK inhibition of chemotaxis in response to chemokines and chemoattractants, implies that there are multiple pathways leading to leukocyte migration. It also highlights the fact that that even though different chemokine receptors can couple to common signalling pathways, there is sometimes redundancy with regards to the importance of those pathways in eliciting a migratory response.

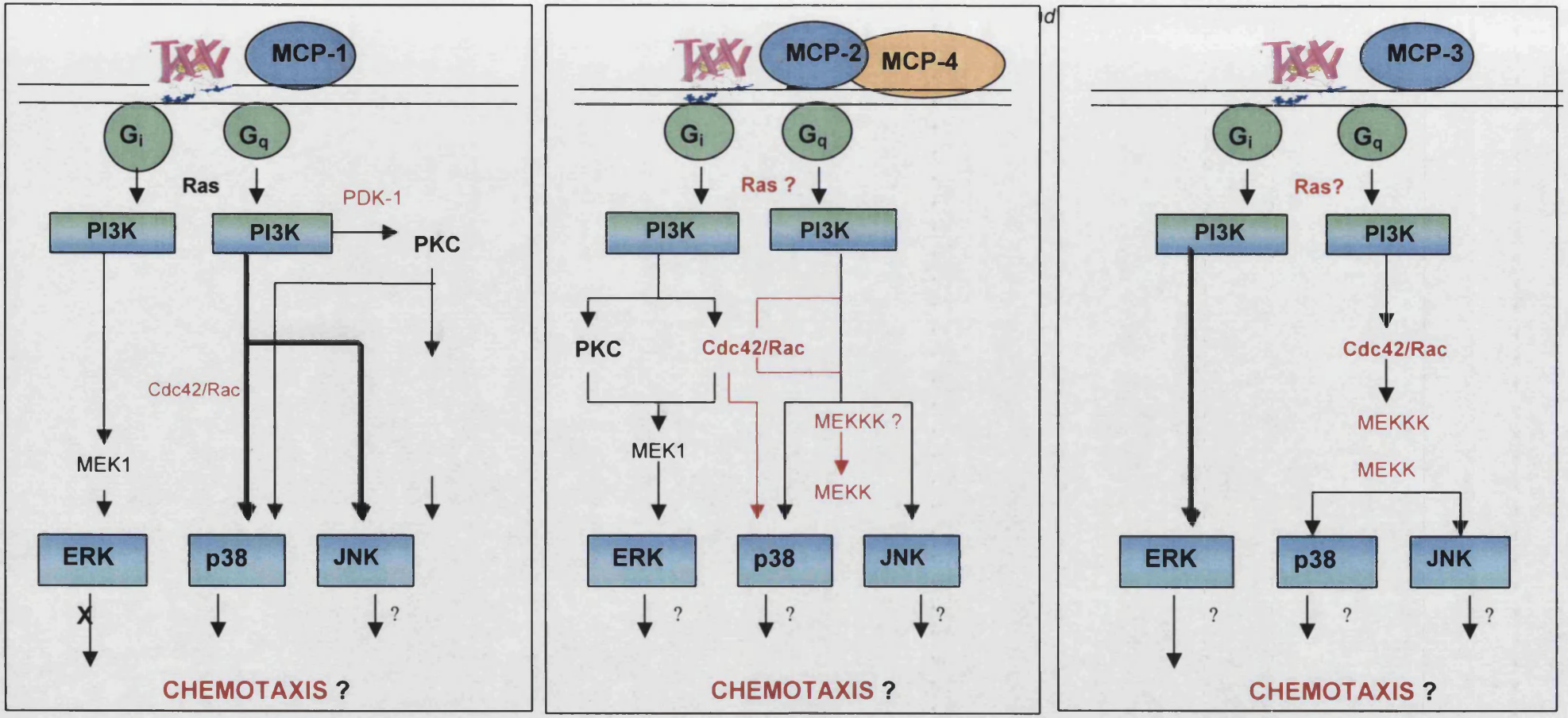
As the roles of p38 and JNK were not assessed in our functional assays, it was impossible to conclude the precise role of each MAPK in this functional response. Nevertheless, considering our data in hand with recent literature, events leading to functional response can be postulated. As discussed earlier, Ashida *et al* characterised the divergence in MAPK signalling in MCP-stimulated THP-1 cells and attributed its functional consequence. These data indicate that two distinct MAPKs

regulate two dependent signalling cascades leading to integrin activation and chemotaxis induced by MCP-1 in THP-1 cells. However, application this hypothesis to our system could only be validated by conducting further functional and biochemical experiments. The use of inhibitors and/or molecular constructs against p38 and JNK would be required to elucidate their specific role in CCR2-mediated cell migration.

Legend for Figure 5.20 (over page). Hypothesised models of CCR-2 mediated MAPK activation by MCP-1, -2, -3 and -4 in THP-1 cells

MCP-1, -2, -3 and -4, Monocyte chemotactic protein-1, -2, -3, -4; G_i, inhibitory G protein: G_q, PLC-specific G protein; PI3K, phosphatidylinositol 3-kiase; Cdc42, Rho family GTPase; Rac, Rho family GTPase; MEKKK, MAPK/ERK kinase kinase kinase; MEKK,MAP K/ERK kinase kinase; MEK1, MAPK/ERK.

Red lines indicate 'hypotheised' signalling event. **Black lines** indicate events characterised during this study.



MCP-1

MCP-1 activates ERK-1/2 and JNK-1/2 via a mechanism that is partially sensitive to PTX, thus inferring differential regulation of G protein subunits. p38 and JNK-1/2, but not ERK-1/2 are regulated by a PKC-dependent mechanism. In the case of JNK-1/2 activation this PKC involvement could be PDK-1-modulated. PI 3-kinase regulates ERK, p38 and JNK and in the case of ERK-1/2 this is a MEK-driven event. The roles of p38 and JNK in MCP-1-stimulated chemotaxis were not assessed, but it appears that ERK-1/2 is not required for cell migration.

MCP-2 and MCP-4

MCP-2 and MCP-4 exhibit similar signalling behaviour in the activation of MAPKs. Coupling of CCR2 to ERK-1/2 is PTX-sensitive, yet PTX-resistant in the activation of JNK-1/2. This G_i-linked pathway to ERK initiated by both MCP-2 and -4 appeared to be PKC-dependent and driven by MEK1. Although p38 and JNK were clearly activated by these ligands, the pathways were not defined. PI3-K appears to play a minor role in this cascade.

MCP-3

MCP-3, like the other CCR2 ligands exhibits heterogeneity its coupling to ERK-1/2 and JNK-1/2. Inhibition of PKC does not affect ERK-1/2 activation and indicates that it does not have an active role. PI3-kinase, on the other hand appears to be a pivotal enzyme in this cascade. It is conceivable that Cdc42 or Rac mediates this PI3-K-dependent activation of ERK-1/2. A similar pathway could well be in place for the activation of p38 and JNK.

Figure 5.20. Hypothesised models of CCR-2 mediated MAPK activation by MCP-1, -2, -3 and -4 in THP-1 cells

Section 6: Results.

The role of phospholipase D in MCP-1-medited signal transduction

Rationale

The interaction of extracellular signal molecules with cell surface receptors quite often activates the phospholipase-D (PLD) and mediates the hydrolysis of phosphatidylcholine and other phospholipids, generating phosphatidic acid and choline. PLD activation and the subsequent liberation of its products is now recognised as an important component of many cellular processes including vesicular trafficking, cytoskeletal dynamics and signal transduction (Billah *et al* 1990: Cook and Wakelam, 1992; Coley *et al*, 1997). To date, only one group has reported the activation of PLD by chemokines in T lymphocytes and the Jurkat T cell line (Bacon *et al*, 1995, 1998), however, its significance is speculative and features of its regulation remain to be elucidated. There is an emergence of evidence linking the PLD and PI3-kinase pathways their PtdIns(3,4,5)P_3-modulation of ADP-ribosylation factors (ARFs). ARFs are regulated by PH domain-containing guanine nucleotide exchange factors, ARF-GEFs, such as GRP-1 and ARNO, and it is their interactions with PI3K that has implications for our understanding of PLD regulation and control of cellular processes controlled by chemokines.

In this chapter, the effect of MCP-1 on phosphatidylbutanol and phosphatidic acid accumulation are measured, and the contribution of PI3K to this pathway was assessed using pharmacological inhibitors. The most potent activators of PLD are small G proteins of the Ras superfamily. Several members of the Ras family appear to regulate PLD activity, including most members of the ARF and Rho families, and of these, ARF proteins appear to be the most active direct stimulators of PLD (Hammond *et al*, 1999; Frohman and Morris, 1996). Brefeldin-A (BFA), a golgi-disturbing agent has been shown to abrogate PLD activity in several systems through the inhibition of the GDP-GTP-exchange of ARF proteins. Here, we have investigated the effect of BFA on MCP-1-induced chemotaxis of THP-1 cells. Together, these studies have provided a foundation for future investigations in to the contribution, if any, of the PLD/PI3K pathway in MCP-1-mediated transmigration.

Results

SDF-1 induced a rapid and transient increase in both phosphatidic acid (PA) and phosphatidylbutanol (PtdBut) - the indicators of PLD activation. Figure 6.1 shows the raw counts of titrated product, and as expected basal levels of PtdBut were approximately 10-fold less than PA. Stimulation of cells with 5ng/ml PMA provided a suitable positive control for all experiments. Figure 6.2 shows a similar profile for MCP-1-stimulated THP-1 cells. Again, stimulation induces a time-dependent increase in both PA and PtdBut, although the kinetics for PtdBut appeared to be slower. It transpired from these time-course experiments that the maximum accumulation of titrated products occurred at 5 min post-stimulation, therefore, all subsequent experiments were performed for this length of time.

PLD activity could be induced by MCP-1 at subnanomolar concentrations with a stimulation concentration range of 0.1-10010nM. Maximum activation was observed as being between two- and threefold above the vehicle controls. Concentrations of MCP-1 greater than 10nM failed to induced increases in PtdBut (Figure 6.3 [insert]).

Figure 6.3. The role of PI3-K in MCP-1-stimulated PLD activation was also investigated using the PI3K inhibitor, wortmannin. MCP-1-induced PLD activation was completely abrogated by wortmannin, although as expected, PMA-stimulated cells were only partially affected. These results give a clear indication that in this system, PLD activation is PI3K-dependent.

Considering the emergence of data suggesting that ARF proteins are involved in the regulation of PLD, the effect of ARF-inhibitor, brefeldin A (BFA), was investigated. Figure 6.4 shows that pre-treatment with BFA severely attenuates MCP-1-induced PtdBut accumulation, although there is a modest amount of resistance. Interestingly, when incorporated into a chemotaxis assay, BFA served to potentiate MCP-1-induced cell migration in a concentration-dependent manner.

Figure 6.5. This effect is also observed in response to MCP-2 and MCP-3, however, MCP-4 appears to be the exception to the rule. Here, MCP-4-mediated chemotaxis is drastically reduced in the presence of BFA. The effects of MCP-2, -3 and –4 on PLD activation were not assessed, and so in this regard, characterisation of the PLD pathway awaits further investigation.

Summary of Results

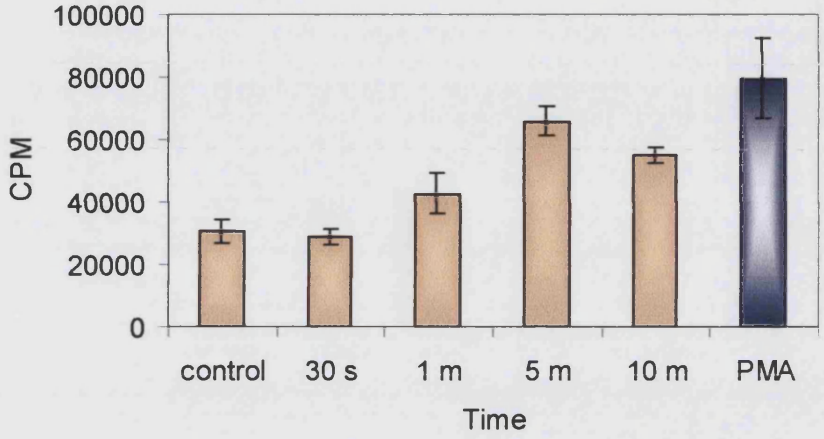
- MCP-1 induces PLD activation in a time-dependent and concentration-dependent manner

- MCP-1 induces PLD activation in a manner that is dependent on both PI3-K and ARF protein(s).

- Chemotaxis induced by MCP-1, -2 and -3 is upregulated by pre-treatment with ARF-inhibitor, brefeldin A, whereas MCP-4-induced chemotaxis is abrogated.

Figure 6.1 Typical values for the separation and analysis of phosphatidic acid and phosphatidylbutanol in chemokine-stimulated cells.

A) Accumulation of PA



B) Accumulation of PtdBut

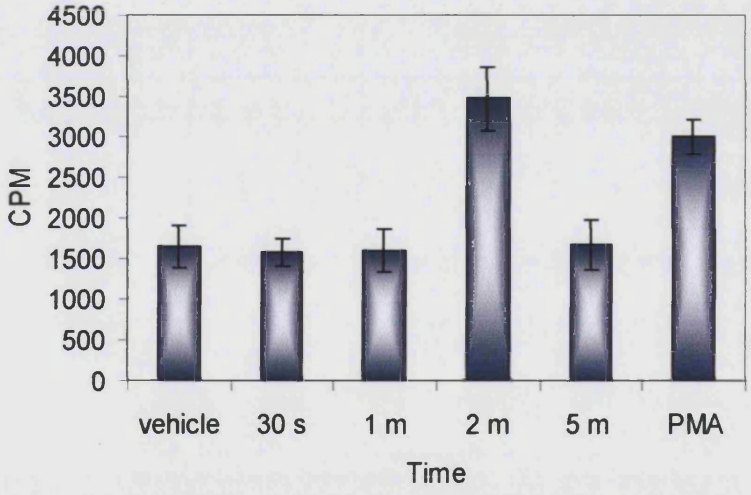
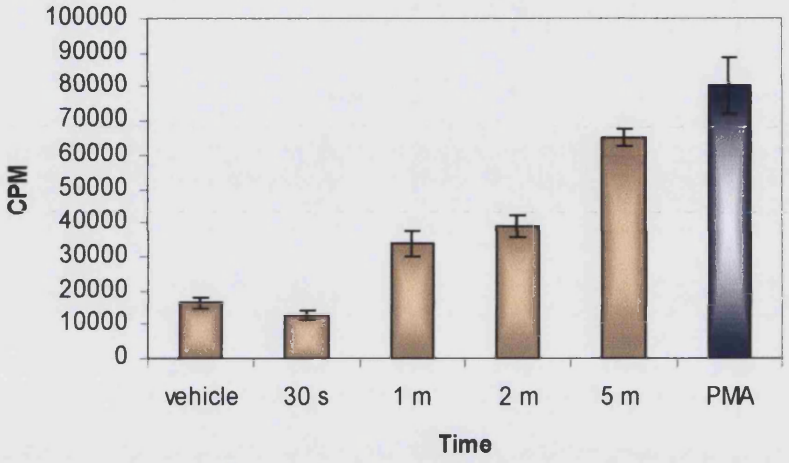


Figure 6.1. Typical values for the separation and analysis of phosphatidylbutanol (Ptdbut) and phosphatidic acid (PA) in chemokine-stimulated cells. SDF-1-stimulated Jurkat cells were initially used as a model for the analysis of **A)** PtdBut and **B)** PA generation in response to chemokines. Jurkat cells ($1x10^7$) were metabolically labelled with $50\mu C_i$ ^{3}H-palmitate for 3 hours and resuspended in **A)** RPMI, **B)** RPMI plus 30mM butanol. Cells were stimulated with 10nM SDF-1 for the indicated times, quenched, and subjected to organic extraction. Extraction, TLC and analysis were performed as described in 'Materials and Methods'. Each histogram represents the mean ± SEM increase in [^{3}H]PA/ [^{3}H]PtdBut (cpm) over background from three independent experiments performed in duplicate.

Figure 6.2. Effect of MCP-1-stimulation on phosphatidylbutanol and phosphatidic acid generation in THP-1 cells.

A) Accumulation of PA



B) Accumulation of PtdBut

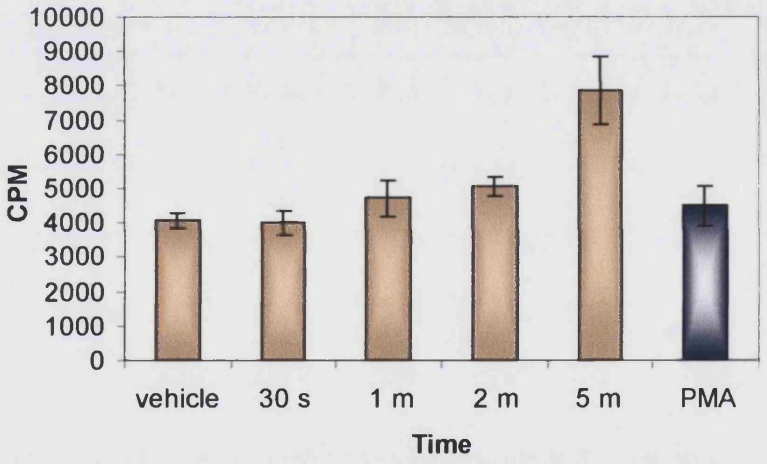


Figure 6.2 Effect of MCP-1-stimulation on phosphatidylbutanol (Ptdbut) and phosphatidic acid (PA) generation in THP-1 cells. THP-1 cells ($1x10^7$) were metabolically labelled with $50\mu C_i$ ^3H-palmitate for 3 hours and resuspended in **A)** RPMI, **B)** RPMI plus 30mM butanol. Cells were then stimulated with 1nM MCP-1 for the indicated times, quenched, and subjected to organic extraction. TLC and analysis of extracted samples were performed as described in 'Materials and Methods'. Each histogram represents the mean ± SEM increase in [^3H]PA/ [^3H]PtdBut (cpm) over background from three independent experiments performed in duplicate.

Figure 6.3 Effect of wortmannin on MCP-1-induced PLD activity

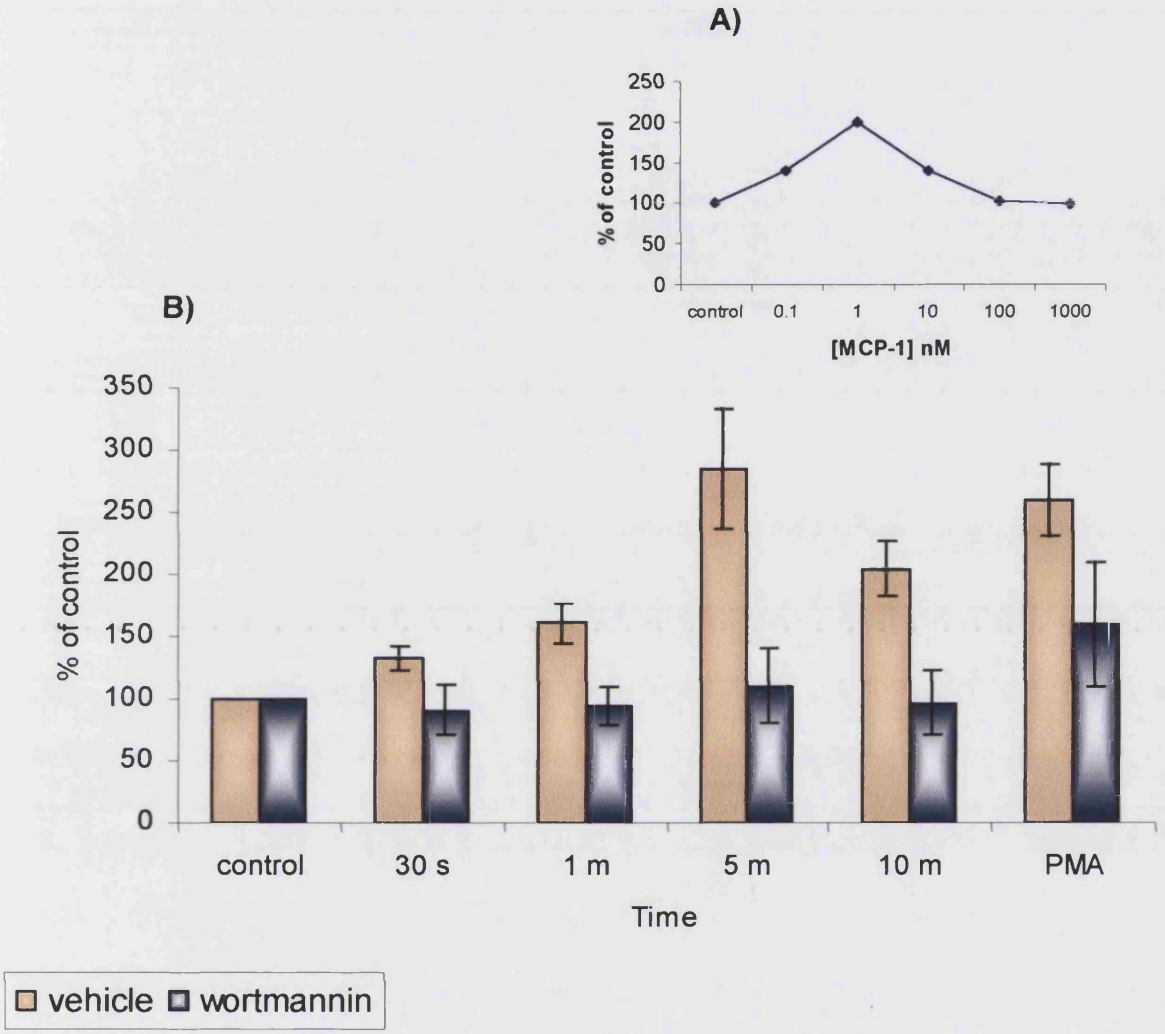
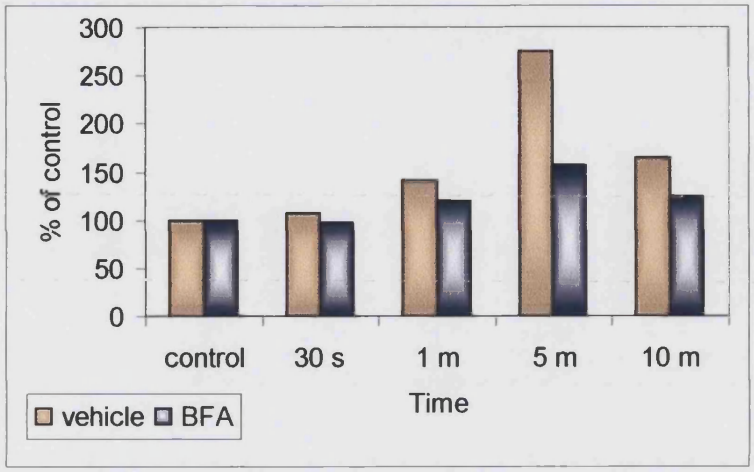


Figure 6.3 Effect of wortmannin on MCP-1-induced PLD activation.THP-1 cells ($1x10^7$) were metabolically labelled with $50\mu C_i$ ^{3}H-palmitate for 3 hours and resuspended in RPMI (30mM butanol) and either pretreated with vehicle (0.01% DMSO) or 10nM wortmannin for 10 m. Cells were then stimulated with 1nM MCP-1 for the indicated times, quenched, and subjected to organic extraction. TLC and analysis of extracted samples were performed as described in 'Materials and Methods'. Each histogram represents the mean ± SEM percentage increase in [^{3}H]PtdBut over background from three independent experiments performed in duplicate. The insert **(A)** represents the demonstrates the concentration-dependency of the reaction at 5 m post-MCP-1 stimulation. Each point represents the mean percentage increase of [^{3}H]PtdBut over background from two independent experiments performed in duplicate.

Figure 6.4 Effect of Brefeldin-A on MCP-1-induced PLD activation and chemotaxis

A)



B)

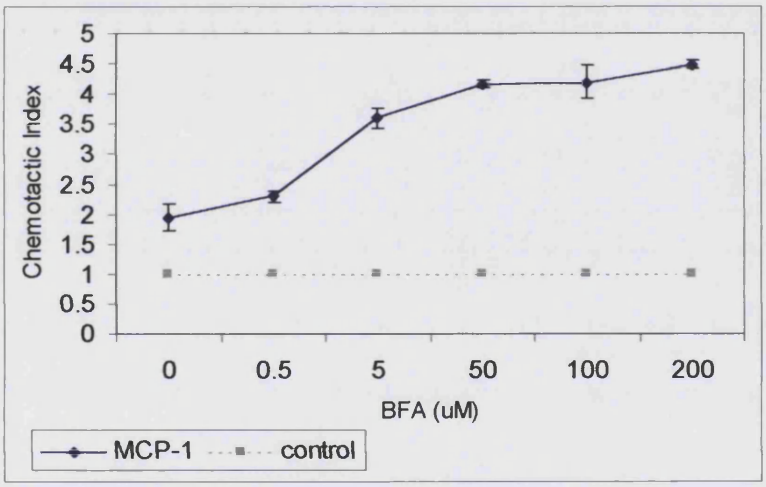


Figure 6.4 A) Effect of Brefeldin-A on MCP-1-induced PLD activation. THP-1 cells ($1x10^7$) were metabolically labelled with $50\mu C_i$ ^{3}H-palmitate for 3 hours and resuspended in RPMI (30mM butanol) and either pretreated with vehicle (0.01% DMSO) or 5μM for 30 m. Cells were then stimulated with 1nM MCP-1 for the indicated times, quenched, and subjected to organic extraction. TLC and analysis of extracted samples were performed as described in 'Materials and Methods'. Each histogram represents the mean percentage increase in [^{3}H]PtdBut over background from three independent experiments performed in duplicate. **B) Effect of Brefeldin-A on MCP-1-mediated chemotaxis of THP-1 cells.** 1 x 10^5 THP-1 cells per point were pre-treated for 15 minutes at 37°C with either vehicle (0.01% DMSO) or Brefeldin-A. After incubation, cells were subjected to chemotaxis towards MCP-1 (1nM) in a 96-well Neuroprobe™ chamber for 3 hours. Migration was determined as described in 'Materials and Methods'. Results are expressed as a Chemotactic Index (C:I): the ratio of stimulated over basal migration. Data represent the mean ± S.EM of duplicate measurements. Results are representative of three independent experiments.

Figure 6.5. Effect of Brefeldin-A on MCP-2, -3 and -4-mediated chemotaxis of THP-1 cells

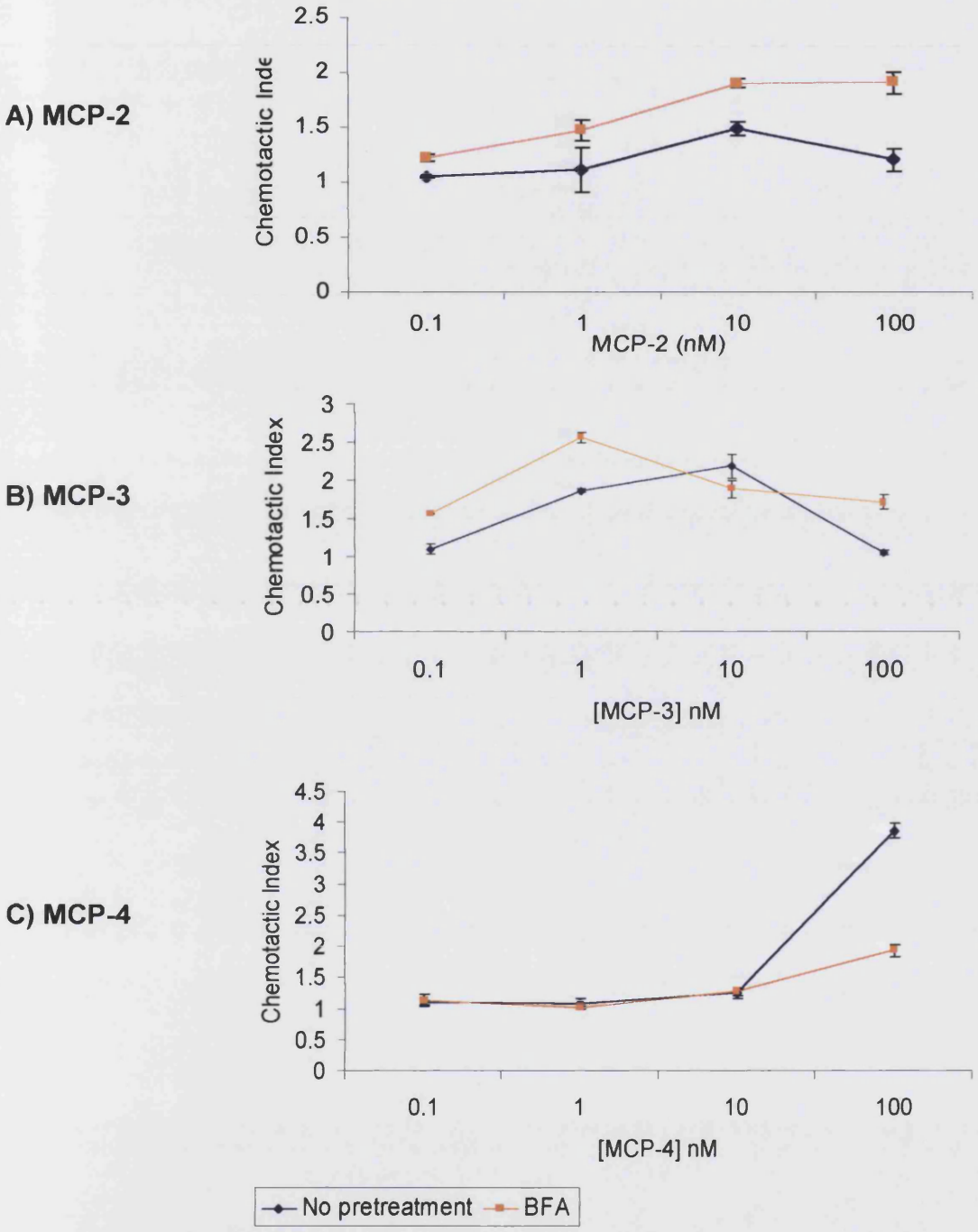


Figure 6.5 Effect of Brefeldin-A on MCP-2, -3 and -4-mediated chemotaxis of THP-1 cells. 1 x 10^5 THP-1 cells per point were pre-treated for 15 minutes at 37°C with either vehicle (0.01% DMSO) or 5μM Brefeldin-A. After incubation, cells were subjected to chemotaxis towards **A)** MCP-2 (10nM), **B)** MCP-3 (10nM) or **C)** MCP-4 (100nM) in a 96-well Neuroprobe™ chamber for 3 hours. Migration was determined as described in 'Materials and Methods'. Results are expressed as a Chemotactic Index (C:I): the ratio of stimulated over basal migration. Data represent the mean ± S.EM of duplicate measurements. Results are representative of three independent experiments.

Section 6: Discussion

The role of phospholipase D in MCP-1-medited signal transduction

We have measured phosphatidic acid (PA) and phosphatidylbutanol (PtdBut) in response to chemokine stimulation. As discussed in Section 1, the measurement of PtdBut accumulation is the definitive test for PLD activation, in that the unique transphosphatidyl reaction catalysed by PLD is only detectable in the presence of a primary alcohol. In directly measuring PA in the absence of an alcohol, signalling pathways other than PLD could be responsible to the generation of this product, such as *de novo* synthesis by DAG-kinase or acylation of glycerol-3-phosphate. In addition, the PA product is susceptible to further metabolism to DAG and lyso-PA, whereas the PtdBut is metabolically stable (Lisovitch *et al*, 1999). PLD activation is beginning to emerge as an important enzyme molecule in chemokine signalling, however, it is also recognised that the measurement of PA is of equal importance in that this lipid, regardless of its source, is now recognised as an important intracellular messenger. For reviews see English, 1996.

MCP-1: An example of chemokine-induced PLD activation

Bacon *et al* have previously shown the potent activation of PLD by chemokines in T lymphocytes. We have added to these findings, and have shown that MCP-1-induced activation of PLD occurs at subnanomolar concentrations and is dependent on PI3K. MCP-1-induced PLD activation is consistently maximal at 1nM, which is pleasing since this concentration corresponds to the optimal dose required for chemotaxis. SDF-1-stimulation of Jurkat cells also induced a time-dependent increase in both PA and PtdBut, and thus we have clearly demonstrated in two separate systems that CC and CXC chemokines can induce detectable PLD activation.

Linking PI3K and PLD

The observation that MCP-1-induced PLD activation occurs downstream of PI3K comes as no surprise since there is a wealth of information suggesting a functional link between these two enzymes. Studies using PI3K inhibitors have suggested, as a possible underlying mechanism, that the products of PI3K, namely PtdIns(3,4)P_2 and PtdIns(3,4,5)P_3 regulate the activity of the afore mentioned small GTPases, ARF and

Rho which have been shown to be involved in PLD activation. This could well explain the wortmannin-induced abrogation of PLD activity in response to MCP-1.
Both PLD and PI3K are regulated by receptor tyrosine kinases and both enzymes appear to be modulated by Ral A, the downstream target of Ras in PDGF- and EGF-stimulated cells (Lucas *et al*, 2000 Slaaby *et al*, 1998). There is also evidence of PLD2 becoming tyrosine phosphorylated on formation of a complex with the EGF receptor (Lu *et al*, 2000), and PLD1 is thought to cooperate with PI3K in the translocation of GLUT4 between plasma membranes and intracellular vesicles in insulin-stimulated cells (Emoto *et al*, 2000).

In contrast, there is a wealth of information implicating PLD upstream of PI3K, and although we have shown that PLD activation in our system is dependent on PI3K, consideration of PLD as an upstream activator may help us to understand the interaction between these pathways.
It has been shown that anionic phospholipids, such as PA can bind to the p110 regulatory subunit of class I PI3Ks. Banno *et al* have highlighted the possibility that in sphingosine-1-phosphate-stimulated cells, PA derived from PLD activation can participate in the recruitment of PI3K to the plasma membrane. The PA-stimulated enhancement of tyrosine phosphorylation in several cell types has been recognised for sometime, however, a more recent study has demonstrated that exogenous PA induces tyrosine phosphorylation of the p85 regulatory subunit of PI3K in neutrophils (Siddiqui *et al*, 1997, 2000). We could therefore speculate that PA-dependent tyrosine phosphorylation may be involved in recruitment to the plasma membrane and stimulation of the PI3K in leukocytes.
In retrospect, it would have been interesting to clarify the effect (if any) of PLD on PI3K activation in this system. Perhaps by incorporating a primary alcohol (versus secondary alcohol) into the stimulation buffer, followed by an *in vitro* lipid kinase assay of a PI3K immunoprecipitate. Alternatively, one could simply assess the effects of this inhibition on PKB phosphorylation. The primary alcohol would serve as a phosphatidyl group acceptor in the PLD-catalysed transphosphatidylation reaction, and inhibit PA/lyso-PA –mediated activation of PI3K.

PLD activation: The requirement of GTP-binding proteins

Considering the notion that ARFs may well be involved in the observed PLD activation, we attempted to investigate the role of these proteins in MCP-1-stimulated chemotaxis of THP-1 cells. Although we cannot deduce the functional significance, we can confirm that PLD activation in THP-1 cells conforms to the standard

requirements for activation of cofactor ARF. Preliminary studies have been carried out to further investigate the role this protein in migration. For this, we used brefeldin A (BFA) ,a fungal metabolite originally isolated from *Penicillium brefeldianum* by Haerri *et al.* The mode of action of this inhibitor is thought to be through the disruption of vesicular and Golgi apparatus, as well as the inhibition of guanine nucleotide exchange factor (GNEF) activity on ARF proteins. In many studies, the use of BFA has clearly indicated the biochemical and functional importance of intact Golgi and ARF proteins in cellular processes. This suggests that BFA is a broad-spectrum inhibitor, and is not exclusively selective to ARF proteins – Thus, caution should be taken when interpreting these results.

It stands to reason that ARF-mediated activation of PLD should be inhabitable by BFA, since BFA inhibits the binding of ARFs to the Golgi membrane. Indeed the abolishment of PLD activation by this compound has been observed in several systems, and was reiterated in this system. Although a good indicator, pre-treatment with BFA is not the definitive test for PLD activation in that some mammalian cells are resistant to inhibition. For instance in PtK1 cells that have natural resistance to BFA (Ktistakis *et al*, 1991), BFA appeared to have no effect on what was shown to be an ARF-mediated activation of PLD (Ktistakis *et al*, 1995).

By observation that BFA severely abrogated MCP-1-induced PLD activation was the first step in linking ARFs and PLD activation to a functional readout. Due to the fact that there are no commercially available inhibitors of PLD, we used BFA in chemotaxis assays as an indirect method of assessing the role of PLD in cell migration - a preliminary step in linking ARFs and PLD activation to a functional readout. Surprisingly, it was shown that BFA failed to inhibit MCP-1-induced chemotaxis of THP-1 cells. Furthermore, our hypothesis was complicated by the apparent concentration-dependent potentiation of the chemotaxis by BFA treatment. In addition, it was found that MCP-2 and MCP-3-induced chemotaxis was potentiated by BFA treatment, migration induced by MCP-4 was abrogated. This is a clear sign that MCP-4 is unique to the other CCR2 ligands in that it appears to be operating via an exclusive ARF-controlled pathway. As we did not look at MCP-2, -3, and –4-stimulated PtdBut or PA accumulation, the role of PLD in cell migration cannot be speculated.

There were several possible mechanisms by which we expected BFA to inhibit MCP-1-induced chemotaxis. Firstly, by abrogating a potential ARF-mediated activation of

PLD. Also, there is confounding evidence that the disruption of Golgi apparatus by BFA blocks cell polarisation and inhibits directed cell migration. For example, in Swiss 3T3 fiboblasts BFA totally abrogated PMA-directed migration by disturbance of psuedopodial activity and lamellipodia formation – morphological prerequisites of cell migration (Bershadsky *et al*, 1994).

As previously mentioned, it possible that the THP-1 cell line exhibits a resistance to BFA, and like PtK1 cells, are not biochemically or morphologically modified by the compound. It is also noteworthy that of all the six known mammalian ARF proteins, only ARF1 has been found sensitive to BFA (Liang and Kornfield, 1997). It may well follow that chemotaxis in this system may involve ARF (s) other than the BFA-sensitive ARF1. Although we have confirmed that MCP-1-stimulated PLD activation is modulated by ARF(s), it is conceivable that PLD does not play a role in chemotaxis. Hence, attenuation of this pathway by BFA would not be detectable in this assay.
In addition, considering that we did not assess any other readout in response to BFA, it cannot be confirmed that the compound was effective in the modification of endogenous ARF proteins as was assumed. However, the action of the toxin was evident by light microscopy since treated cells were less spherical than those treated with the vehicle (data not shown), and is an indication that BFA was having the desired cellular response.

Since a significant amount of literature suggestive of a direct role of Rho proteins in receptor-mediated PLD activation has been published in recent years, the involvement of Rho in this system should also be considered. For example, insulin induces the translocation of ARF and Rho to the plasma membrane of rat adipocytes and that activation of PLD by insulin correlates with the activation of Rho proteins (Karnam *et al*, 1997). In hand with BFA, the use of *Clostridium botulinum* C3 exotxin, an inhibitor of Rho function, would have been a useful way in delineating the function of these small G proteins in PLD activation.

Given the idiosyncrasies involved in using BFA as an inhbitor of ARF proteins, the resistance of MCP-1-stimulated chemotaxis of THP-1 cells can be rationalised. However, the observed potentiation of chemotaxis by this compound is difficult to explain. The ARF proteins have been implicated in the retrograde transport of proteins between Golgi and endoplasmic reticulum (Dinter and Berger, 1998), which makes MCP-1-induced activation of PLD a likely candidate mechanism for CCR2

downregulation. Given this hypothesis, it follows that inhibition of PLD by BFA would reverse this effect by up-regulating CCR2, and therefore rendering cells more responsive to MCP-1 directed migration. Although plausible, such this theory is wildly speculative, and until further studies are carried out, the pathways mediating this effect remain unclear.

PA has been shown to activate tyrosine phosphorylation, inhibit Ras-GAP, stimulate the recruitment of Raf-1 to cell membranes, induce mobilisation of intracellular calcium, and appears be more potent than DAG in phosphatidyl-inositol bisphosphate (PIP_2)-specific PLC activation. Furthermore, PA may potentiate the function of kinases, G-proteins, exert transcriptional control, and other mediators that are involved in signal amplification or directly in cellular responses. Although our insight into the intracellular targets of PA is clearly advancing, the absence of selective and potent inhibitors of PLD, are limiting the experimental approaches that can be employed for implicating PLD in cell function.

Macrophages, PLD and atherosclerosis: A clinical perspective.

So how does the activation of the PLD pathway by MCP-1 fit into the pathogenesis of atherosclerosis and other inflammatory disorders? As discussed in Section 1, much of the current interest in the role of chemokines in atherogenesis stems from MCP-1 and CCR2 knockout mice. However, while it is clear that this receptor-ligand interaction is involved in the initiation of atherosclerosis *in vivo*, it is not clear how MCP-1 effects contributes to the artherogenesis (development of atherosclerotic lesions). CCR2-mediated recruitment of macrophages and the subsequent uptake of oxidised LDLs is an important factor in the development of this disorder, and interestingly, several lines of evidence have implicated oxidised LDLs in the activation of the phospholipase D (PLD) pathway. For instance, it was reported by Natarajan *et al* that in arterial smooth muscle cells, oxidised LDL induced DNA synthesis, and this was highly associated with stimulation of PLD activity.

There are several groups that have identified that atherosclerotic plaque inflammatory proteins such as VCAM-1 or CD59, contain glycosylphosphatidylinositol (GPI) membrane anchors (Terry *et al*, 1993). This has highlighted the possibility that an enzyme associated with atherosclerosis may catalyse the cleavage of the GPI moiety. To date, the only recognised GPI-specifc enzyme is a GPI-specific PLD. Interestingly, GPI-PLD is highly expressed in the majority of myeloid cell lines, and infiltration of such cells is a hallmark of atherosclerotic plaque formation (Xie and

Low, 1994). Furthermore, the cleavage products of GPI-PLD are shown to upregulate macrophage expression of IL-1 and TNFα, the major inflammatory cytokines believed to mediate atherogenesis (Schofield *et* al, 1996; Moyer *et al*, 1991). The fact that we have shown the monocytic cell line, THP-1, to activate PLD in a CCR2-mediated manner is further evidence that this signalling pathway could participate in the pathogenesis of atherosclerosis.

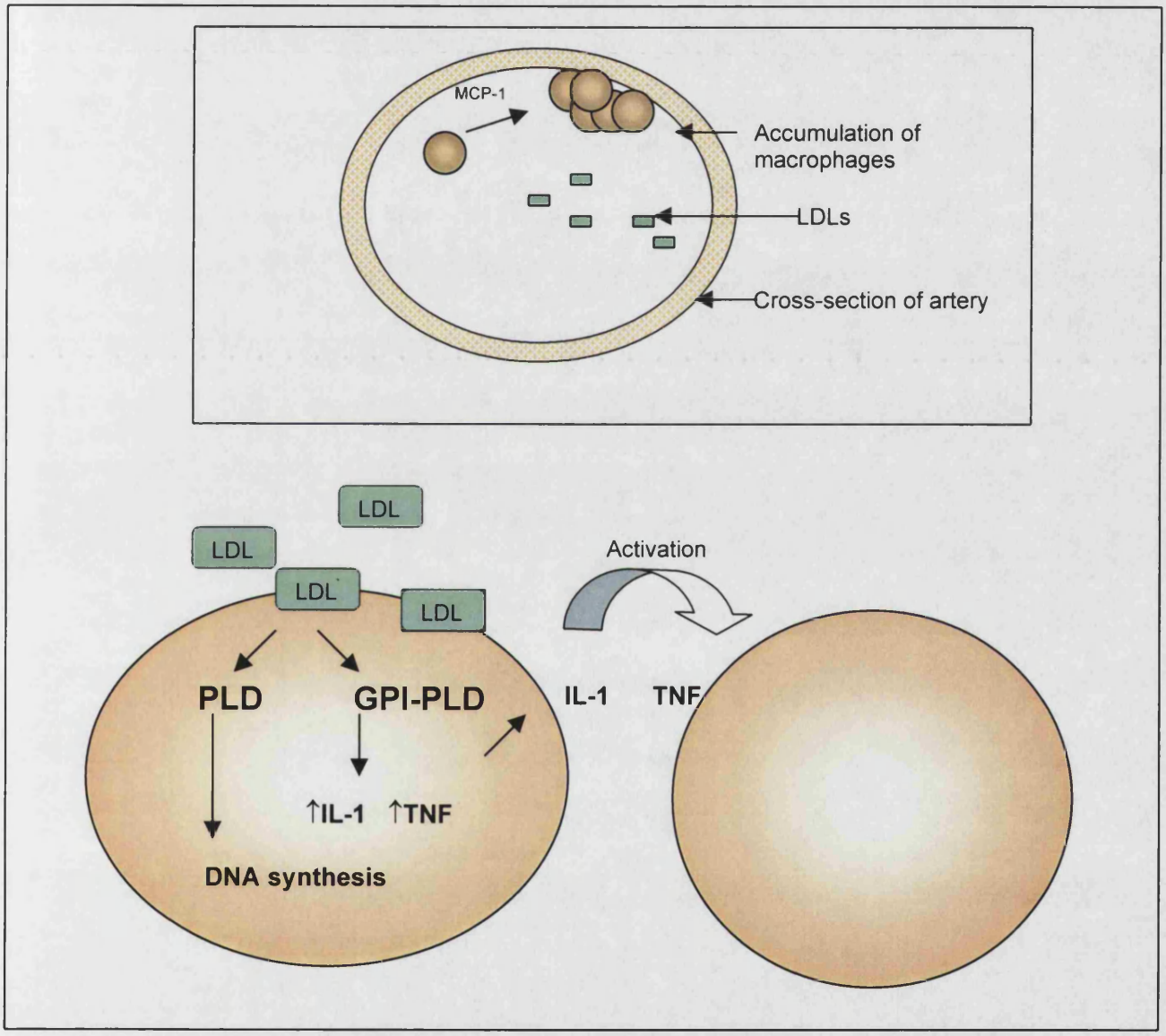


Figure 6.6. **The hypothetic link between CCR2, PLD and LDL in artherogenesis**. . Monocytes are recruited to the artery by MCP-1 secreted from smooth muscles cells. Here, circulating monocytes engage in MCP-1-controlled rolling and adhesion. Following differentiation in to macrophages, oxidised LDLs are taken up and foam cells are formed. LDLs activate PLD that modulates various signalling cascades and DNA synthesis. The cleavage products of GPI-specifc PLD upregulate the expression of IL-1 and TNF-α, (cytokines highly associated with chronic arterial inflammation), and these perpetuate artherogeneis in an autocrine manner.

Future studies

- Key experiments that are outstanding include: assessment of PLD activation in response to MCP-2, -3 and –4, and the role of PI3-K and ARF(s) in these pathways.

- Further delination of the coupling mechanism of CCR2 to the PLD pathway by determining the involvement of tyrosine kinases, PKC, and other small GTPases such as Ras, Rac and Rho.

- Based on studies carried out with neutrophils (English, 1996), it would be interesting to investigate the extracellular effects of PA and other PLD products on macrophage function. This could be achieved by exposing cells to exogenous PA and assessing cellular responses such as actin polymerisation, gene transcription, cell migration.

- Considering the recent interest surrounding ARF-GEF interaction with the lipid products of PI3-K, future studies could involve the investigation of their cellular function. Our group is currently using green fluorescent protein (GFP)- imaging of ARF-GEFs such as ARNO and cytohesin to help characterise the PI3-K-dependent regulation and subsequent PLD activation.

Section 7: Results

Characterisation of signal tranduction events elicited via a novel promiscuous human CC chemokine receptor, D6.

Rationale

So far in this thesis, we have explored the signal transduction mechanisms arising from engagement of the CCR2 receptor by MCP-1, and related family members. For the final chapter, we briefly touch upon a different CC chemokine receptor-ligand pair and presenting preliminary data from macrophage inflammatory protein-1 α (MIP-1α) and its novel promiscuous receptor, D6.

Human D6 is a promiscuous G protein-coupled heptahelical chemokine receptor that is expressed is a wide range of tissues. Although assigned to the CC chemokine family, comparisons of the primary sequence of D6 suggests that it is equally related to the CC and CXC families (Nibbs *et al*, 1997), and so may represent a novel subset of chemokine receptors. Most of the information regarding this receptor comprises expression profiles and binding data, but to date there is no evidence of signalling potential. Here we used cells stably transfected with D6 cDNA in the presence and absence of specifically mutated residues - residues that were noticeably different in D6 compared to all other chemokine receptors. This model was used to evaluate the ability of the D6 receptor to couple to a variety of downstream effectors in an attempt to verify whether D6 is indeed a signalling receptor.

Table 7.1 Affinity of Human and Murine CC chemokines on hD6

Chemokine	Human	Murine
MIP-1α	64nM	920pM
MIP-1β	1.7nM	755pM
RANTES	3.6nM	ND
MCP-1	16.5nM	613nM
MCP-2	768pM	ND
MCP-3	1.3nM	ND
MCP-4	5.97nM	ND
MCP-5	ND	6.3nM
Eotaxin	46nM	30nM

Table 7.2 D6 chemokine receptor plasmids

Plasmids	Comments
Wild type	No mutation. No calcium flux
N92D	The N at position 92 in D6 is D in all other CC receptors (except Duffy). No calcium flux
K142A + E145A	Double mutation. Introduction of a DRYLAIVHA motif into the second intracellular loop (DKYLE-DRYLA), with the overall charge of this region being altered. Modest calcium flux
K142A + E145A + N92	Contains both N92D and K142A+E145A. Modest calcium flux

All plasmids were constructed and transfected by Robert Nibbs, Beatson Institute, Scotland

- HEK-293 cells were either untransfected or stably transfected with one of the four plasmids above.
- 300-19 Pre-B (clones 10 and 13) were either untransfected or stably transfected with wild-type D6.

Results

Figure 7.1. MIP-1α-stimulated wild-type HEK-293 cells did not show a measurable increase in ERK-1/2 phosphorylation, but appeared have constitutive activity of the enzyme. Conversely, the N92D mutant had relatively low basal activity, and upon stimulation with MIP-1α demonstrated a rapid and transient phosphorylation of ERK-1/2. Maximum activity was achieved 1 m post-stimulation and had returned to basal levels after 2 m. Stimulation with PMA for 5 m provided an appropriate positive control for this assay.

The same transfectants were assessed for their ability to induce PI3-kinase activity Both the wild-type D6 and the N92D mutant were assessed for their ability to phosphorylate a well-characterised downstream effector of PI3-kinase, protein kinase

B (PKB). Figure 7.2. When stimulated with 10nM MIP-1α a rapid and transient phosphorylation of PKB was observed in both wild-type and N92D transfectants with maximum activity observed 1 m post-stimulation. In the case of N92D, the kinetics appeared to be slightly slower and more sustained than that of the wild-type.

Although the phosphorylation of PKB implied that PI3-kinase was being activated in this system, it was necessary to clearly identify the isoforms involved in D6-mediated signalling. Two cell lines (HEK-293 and 300-19) containing the D6 cDNA were screened for the G protein-coupled receptor-specifc PI3K, p110γ, by western blotting resting cells with an anti-p110γ monoclonal antibody. Figure 7.3A shows the relative expression of PI3Kγ in the D6 cell models compared to that of Jurkat and THP-1 cells. Interestingly, whereas the HEK-293 cells screened positive for this isoform, there was no evidence of p110γ expression in the pre-B cell line, 300-19.

Based on these observations, the p110γ-positive HEK-293 D6 tranfectants were subjected to investigation of associated lipid kinase activity under *in vitro* conditions (as explored in Section 4). Such *In vitro* lipid kinases assays demonstrated that stimulation of both HEK-293 wild-type and N92D D6 transfectants with MIP-1α (100nM) resulted in a time-dependent increase in p110γ activity. In the wild-types, increase in p110γ activation, although modest, peaked at 1 m and declined to below base-line levels after 15 m. The N92D mutants appeared to be more responsive to MIP-1α stimulation and demonstrated a greater increase in p110γ activity. Maximum activity was observed at 1 m post-stimulation and had returned to near basal levels after 10 m (Figure 7.3B).

In a crude attempt to investigate the role of phospholipase D (PLD) in D6-mediated signalling, the generation of phosphatidic acid was measured in response to MIP-1α stimulation (Figure 7.4). In the HEK-293 D6 transfectants generation of phosphatidic acid was evident after 30 s post-stimulation, maximal at 5 m (~2-fold increase) and had almost returned to basal levels after 10 m.

As expected, the untransfected HEK-293 cells did not respond to stimulation. In the 300-19 D6 cell model, levels of PA increased to 286% of control upon stimulation with MIP-1α. In this cell line, although maximum activity was greater, the kinetics of PA accumulation were slower and more sustained than observed in the HEK-293 cells since levels of PA generation were over 200% of control after 10 m post-stimulation.

In the HEK-293 transfectants, the effects of the N92D and the DKYLE-DRYLA mutations on the D6-mediated phosphatidic acid were compared to that of the wild-type (Figure 7.5). Although maximum PA generation was attained with the wild-type (268% of control), the N92D and the DKYLE-DRYLA mutants also permitted activation. The DKYLE-DRYLA mutant produced the weakest response, peaking at ~160% of control. In short, these mutations served to reduce the efficiency of D6-coupling to the PA pathway.

Unfortunately, due to constraints of time, the triple mutant was not introduced into any of the above experiments.

Figure 7.1 Effect of MIP-1α on ERK-1/2 activation in HEK-293 cells stably transfected with D6 cDNA

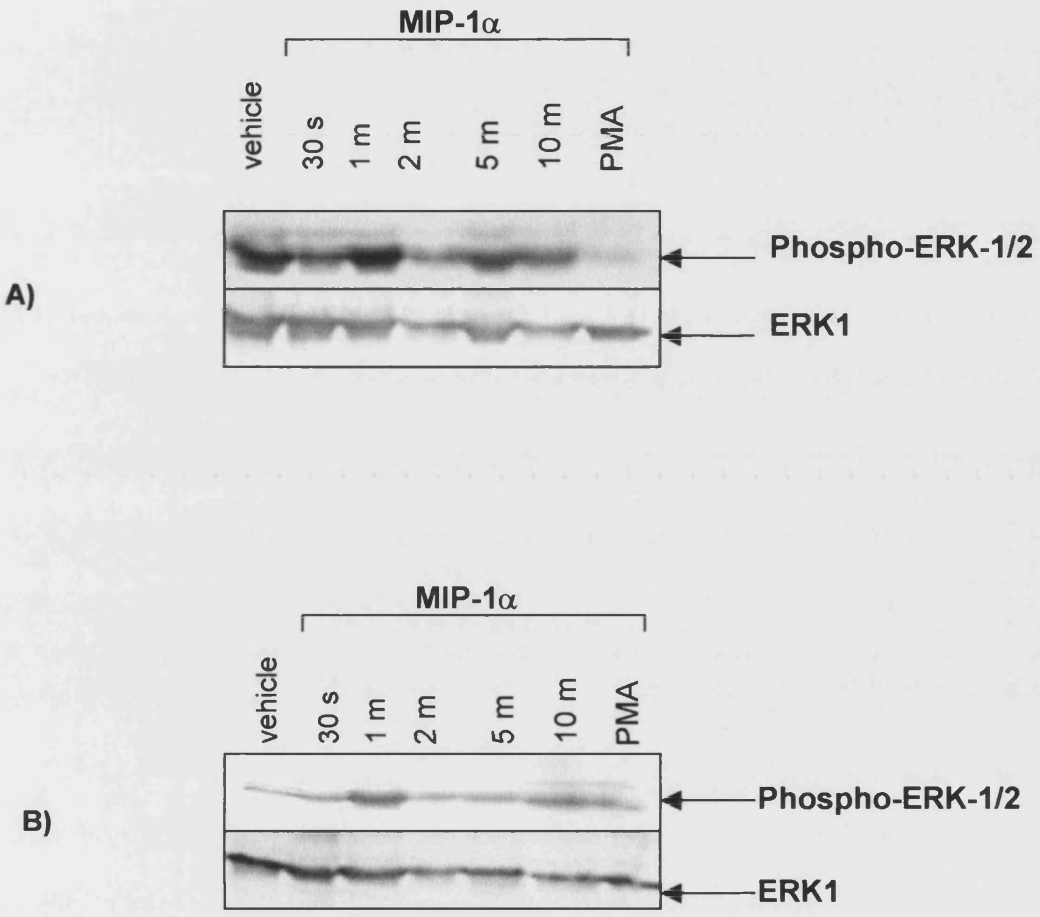


Figure 7.1 Effect of MIP-1α on ERK-1/2 activation in HEK-293 cells stably transfected with D6 cDNA. Pools of geneticin-resistant HEK-293 cells were stably transfected with pcDNA3 containing D6 (A) wild type, B) N92D mutant. 5 X 10^5 cells/point were then stimulated with either vehicle (0.05% BSA) or 10nM MIP-1α for the indicated times . Whole cell lysates (cell equivalents of 2.5 x 10^5 per lane) were resolved separately by SDS-PAGE, electrophoretically transferred to a nitrocellulose membrane and immunoblotted with either anti-phospho-ERK-1/2 or pan ERK1 (to verify equal loading of proteins). The results are from one experiment but are representative of at least one other.

Figure 7.2. Effect of MIP-1α on PKB activation in HEK-293 cells stably transfected with D6 cDNA

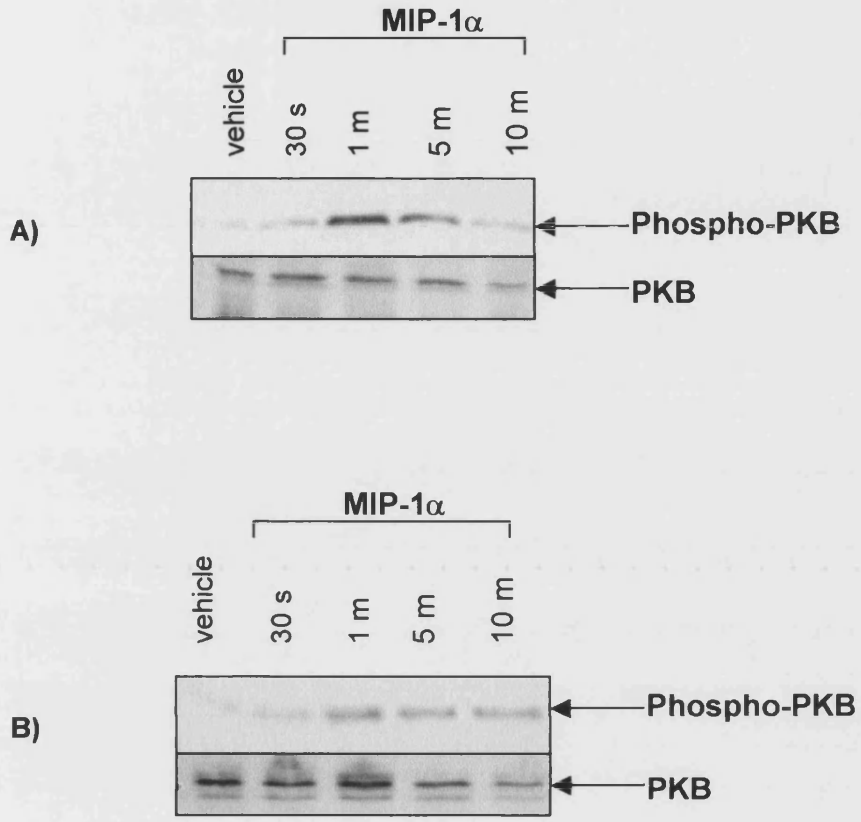


Figure 7.2. Effect of MIP-1α on PKB activation in HEK-293 cells stably transfected with D6 cDNA. Pools of geneticin-resistant HEK-293 cells were stably transfected with pcDNA3 containing D6 (A) wild type, B) N92D mutant. 5 X 10^5 cells/point were then stimulated with either vehicle (0.05% BSA) or 10nM MIP-1α for the indicated times . Whole cell lysates (cell equivalents of 2.5 x 10^5 per lane) were resolved separately by SDS-PAGE, electrophoretically transferred to a nitrocellulose membrane and immunoblotted with either anti-phospho-PKB or pan PKB antibodies (to verify equal loading of proteins). The results are from one experiment but are representative of at least one other.

Figure 7.3. Verification of expression and activity of Class 1_B PI3K isoform, PI3Kγ in HEK-293 cells stably transfected with the D6 receptor.

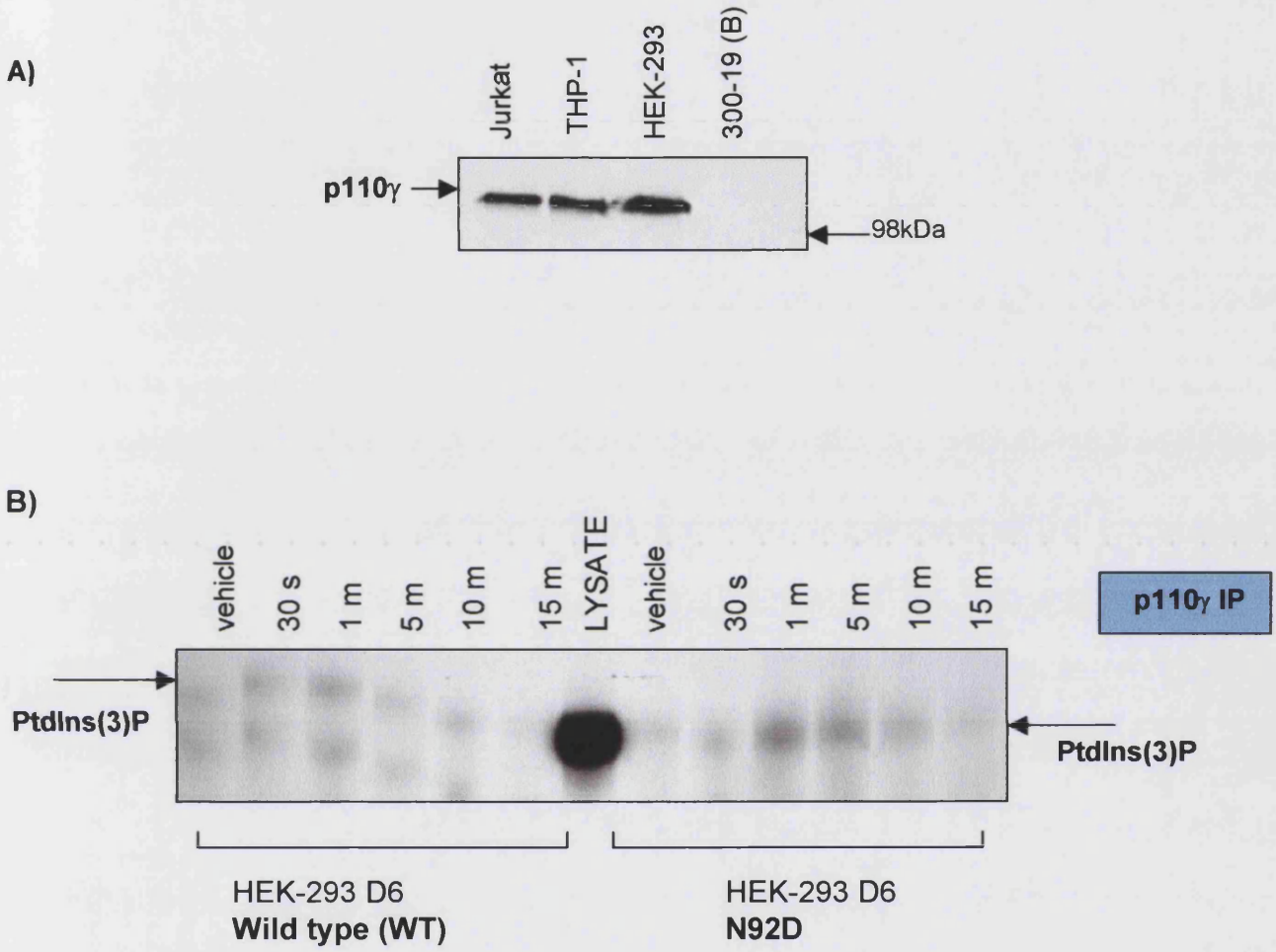
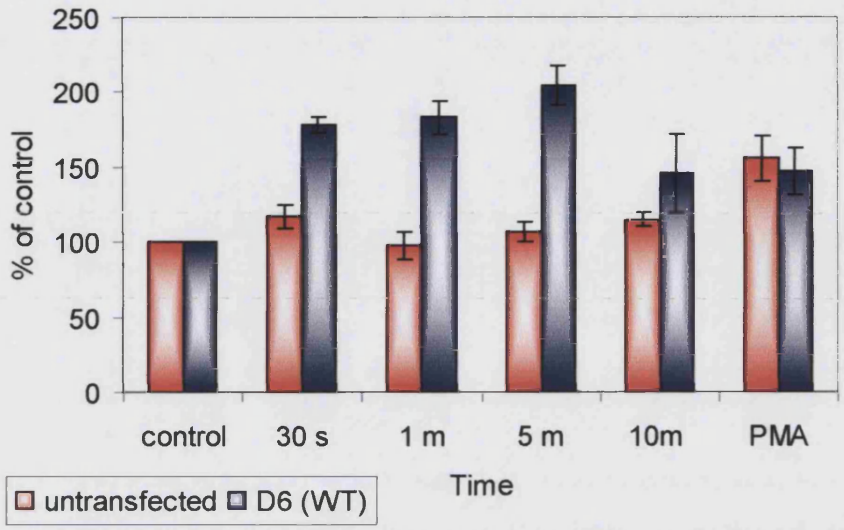


Figure 7.3. A) Expression of p110γ in HEK-293 cells. Resting Jurkat, THP-1, HEK-293 and 300-19 Pre-B cells were lysed, resolved separately by SDS-PAGE (2.5 x10^5 cell equivalents per lane), and electrophoretically transferred to a nitrocellulose membrane as described in 'Materials and Methods'. Lysates were immunoblotted with an anti-p110γ mAb. Results are from one experiment and are representative of another 2 independent experiments **B) *In vitro* lipid kinase assay of MIP-1α-stimulated HEK-293 cells stably transfected with the D6 receptor.** 1x10^7 HEK-293 cells/point (wild type and N92D mutant) were stimulated at 37°C with 10nM MIP-1α for the indicated times. Cells were lysed, and lysates subjected to immunoprecipitation with anti-p110γ monoclonal antibody. The washed immunoprecipitates were analysed for PI kinase activity using PI as a substrate. Extraction and TLC separation of the lipid products were performed as described under 'Materials and Methods'. Lipids were detected by exposure to film at -70°C. The data is representative of three separate experiments.

Figure 7.4. Generation of phosphatidic acid in MIP-1α-stimulated human D6 transfectants

A) HEK 293



B) Pre-B (300-19)

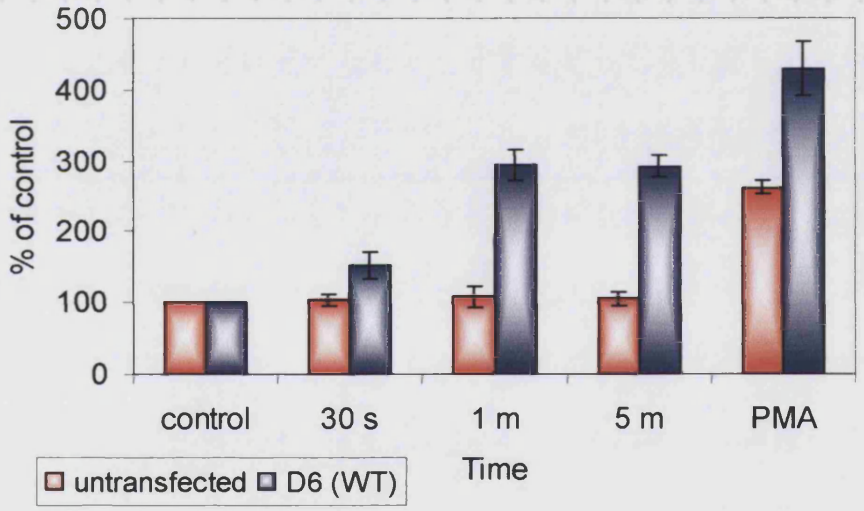


Figure 7.4. Detection of phosphatidic acid generation in human D6 transfectants. Pools of **A)** geneticin-resistant HEK-293 cells and **B)** Pre-B (300-19) cells transfected with pcDNA3 containing D6 were metabolically labelled with 50μC_i ^{3}H-palmitate for 3 hours and resuspended in RPMI. Cells were then stimulated with 10nM MIP-1α for the indicated times, quenched, and subjected to organic extraction. TLC and analysis of extracted samples were performed as described in 'Materials and Methods'. Each histogram represents the mean ± SEM percentage increase in [^{3}H]-Phosphatidic acid over background from three independent experiments performed in duplicate.

Figure 7.5 Detection of phosphatidic acid generation in HEK-293 cells stably transfected with D6 cDNA.

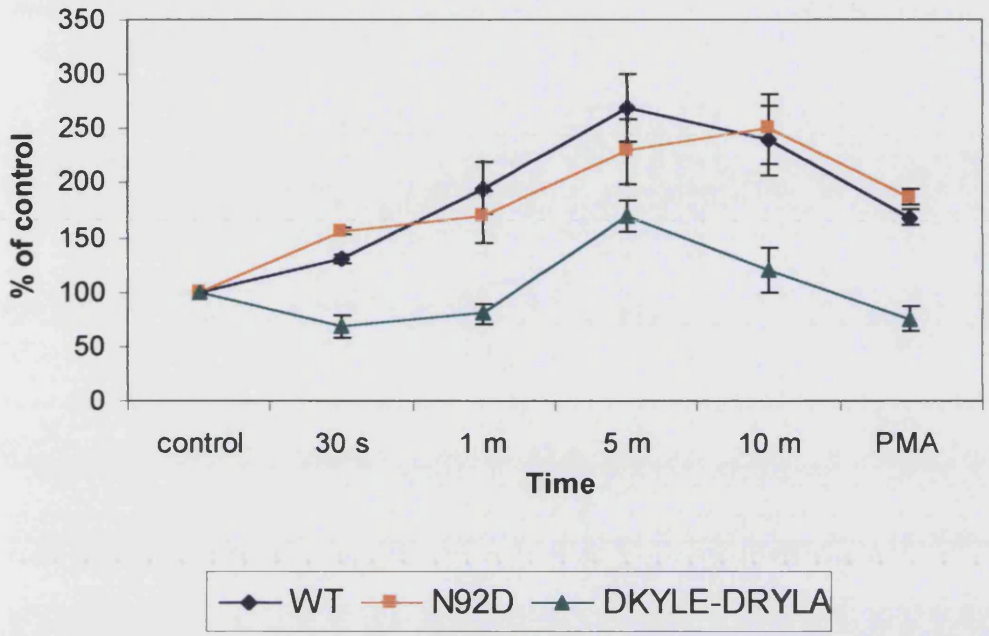


Figure 7.5. Detection of phosphatidic acid generation in HEK-293 cells stably transfected with D6 cDNA. Pools of geneticin-resistant HEK-293 cells transfected with pcDNA3 containing D6 (wild type, N92D and DKYLE-DRYLA mutants) were metabolically labelled with 50μC_i ^{3}H-palmitate for 3 hours and resuspended in RPMI. 1 X 10^7 cells/point were then stimulated with 10nM MIP-1α for the indicated times, quenched, and subjected to organic extraction. TLC and analysis of extracted samples were performed as described in 'Materials and Methods'. Each histogram represents the mean $\pm$ SEM percentage increase in [^{3}H]-phosphatidic acid over background from two independent experiments performed in duplicate.

Section 7: Discussion

Characterisation of signal tranduction events elicited via a novel promiscuous human CC chemokine receptor, D6.

One of the most pertinent questions in the study of chemokine biology is why there is so much redundancy among the ligands and why are many ligands promiscuous with respect to receptor activation (For review see Mantovani, 1999). For instance, as observed in previous chapters, all four monocyte chemotactic proteins (MCPs) interact with CCR2, and at least MCP-2, -3 and –4 also recognise other receptors. It is interesting to note that each chemokine is uniquely active upon different leukocyte populations, and similarly, a given leukocyte population responds to a multitude of chemokines. So what could be the true relevance of the promiscuity observed in the ligand:receptor interactions that occur between multiple members of the chemokine family?

In 1997, Nibbs *et al* reported the identification and characterisation of a novel CC chemokine receptor, human D6. This was shown to be a highly promiscuous receptor that was able to bind the majority of CC chemokines, however, it demonstrated high specificity in that it did not show affinity for members of the C, CXC or CX_3C families. Uncommon to most chemokine receptors, human D6 has not been shown to flux calcium, and so until now, it has been unclear whether this novel receptor is indeed capable of signalling. Addressing these issues will enable us to understand the biochemical and functional role of the D6 receptor and may also extend our understanding of promiscuous chemokine receptors in general. Furthermore, observing the effects of mutation on the signalling capacity of D6 will assist the identification of important microdomains that may be involved in agonist binding and the activation of CC subfamily of chemokine receptors. Although the ligand specificity of D6 is contentious (Bonini *et al*, 1997) full length D6 cDNA expressed in HEK-293 and CHO cells produces the highest affinity murine MIP-1α receptor identified to date (Nibbs *et al*, 1997a). Here, using murine MIP-1α, we have extended our studies to investigate whether D6 is in fact capable of eliciting detectable signal transduction.

Northern blot analysis of several human tissues reveals that D6 is almost exclusively expressed in the placenta with weak expression in the liver, lung and thyroid. Early studies reported that there is only a weak haematopoitic expression of hD6, thus, supporting the observation that hD6 does not facilitate HIV-1 or SIV entry into CD4-expressing cells (Nibbs *et al*, 1997a). In disagreement, RT-PCR studies in our laboratory have clearly shown mRNA expression of this receptor on peripheral blood mononuclear cells, THP-1 and U937 cells (data not shown). Current studies have focused on the observation that D6 is highly expressed on lymphatic endothelial cells and some malignant vascular tumours. Here, it has been suggested that D6 may influence the chemokine-driven recirculation of leukocytes through the lymphatics and modify the putative chemokine effects on the development and growth of vascular tumours (Nibbs *et al*, 2001).

Despite knowledge of ligand binding relationships and tissue localization of D6, the precise role of this receptor in normal and pathologic physiology remains unclear. Based on what is known of other heptahelical membrane receptors, it seems likely that D6 would transmit a signal across the membrane upon chemokine binding, however, D6-dependent signal transduction has not yet been formally reported. This is the first evidence that the wild-type D6 receptor activates the PI3K/PKB pathway and weakly phosphorylates ERK-1/2 in HEK-293 cells, and mediates the generation of phosphatidic acid in both HEK-293 and 300-19 transfectants.

PLD activation in D6 transfectants

Stimulation of D6 transfectants with MIP-1α resulted in accumulation of phosphatidic acid (PA), and was demonstrated in wild-types, N92D and DKY-DRY mutants. As extensively discussed in Section 6, the methodology employed in our studies does not permit us to automatically attribute the generation of PA to the involvement of phospholipase D. That said, PLD-mediated generation of PA is consideration here given that there is a growing body of evidence emerging that establishes the chemokine-induced accumulation of PLD. Bacon *et al*, have reported chemokine-stimulated PLD activation in the Jurkat T cell line, and although the receptor(s) mediating this effect await characterisation, this cell line was shown to express D6. Therefore, in hand with our data, it is tempting to speculate that ligation D6 receptor potently activates PLD. Considering the establishment of potential links between PI3K and PLD in such systems, future studies will be aimed at characterising the of cross-talk between these pathways.

Although commonly considered as a mere lipid intermediate resulting from PLD or DAG-kinase activation, phosphatidic acid *per se* is now suspected to play a role as a

second messenger in several signalling pathways. It appears to be involved in cell proliferation induced by cytokines, membrane trafficking, and cytoskeletal reorganisation. In addition, there is a non-exhaustive list of PA-sensitive signal transduction proteins that further implicate PA as a regulator of cell function. Examples include PKC, Ras, cAMP-phosphodiesterases (Epand and Stafford, 1990; Tsai *et al*, 1989; Grange *et al*, 2000). Apart from it effects on Raf-1 kinase function, little information is available on the functional significance of PA. In this regard, PA has been reported to control membrane recruitment and activation of Raf-1 and as a consequence plays an intrical part in the Ras/MAPK cascade. (Bell *et al*, 1995). Whether the observed PA accumulation was a result of PLD activation or not, it can be concluded that ligation of the D6 receptor initiates the activity of this intermediate and/or second messenger which may be involved in important cellular processes. This is further evidence that this D6, contrary to the literature, is indeed a signal transduction-competent chemokine receptor.

D6 activation to the PI3K/PKB pathway

Phosphorylation of PKB in response to MIP-1α clearly demonstrates the formation of D-3-phosphorylated lipids in this system, and hence the activation of phosphatidylinositol-3-kinase (PI3K). Furthermore, it has been established here that D6 couples to the G protein-associated PI3K as observed from the *in vitro* lipid kinase activity in p110γ immonoprecipitates. Wild-type D6 transfectants produced a modest, time-dependent increase in p110γ in response to MIP-1α-stimulation. These data encourage us to question how the D6 receptor couples to the PI3K-γ given that this receptor appears to be lacking the crucial motif required for G protein interaction. The hD6 cDNA displays 71% identity to the murine D6 receptor, and in common with this protein, it has an alteration in the highly conserved DRYLAIVHA motif in the second cytoplasmic loop that is characteristic of G-protein-coupled seven-membrane-spanning receptors. To date, only a few other heptahelical receptors that lack DRY motifs have been cloned. They include the cAMP receptor in Dictostelium (Klein *et al*, 1988), bride of Sevenless (BOS) in drosophila (Hart *et al*, 1990), and the two, seven-transmembrane-spanning proteins that bear mutations in familial Alzheimer's disease (Rogaev *et al*, 1995; Sherrington *et al*, 1995) The yeast homologue of the heptahelical protein mutated in Alzheimer's disease has been shown to be involved in signalling via the notch pathway (Levitan *et al*, 1995). Thus, there is precedent for heptahelical receptors that signal through pathways

independent of G-coupled proteins. On ligation of the receptor, the D6-specifc DKY motif could interact or "cross-talk" with other membrane components (which could differ depending on cell type) and may permit a novel type of G-protein interaction quite different to the typical GPCR interactions. This would explain the MIP-1α-stimulated p110γ activation observed in the wild-type and N92D D6 transfectants. In retrospect, it would have been useful to assess the pertussis toxin-sensitivity the MIP-1α-stimulated PI3Kinase/PKB pathways, as this would have provided clear evidence of the role of the G protein subunits in D6-mediated signalling.

The Role of D6 – Lessons from DARC.

The scope for possible biological functions of D6 is extremely broad given that there is limited information on this receptor. However, due to the promiscuity, the 'apparent' non-signalling status, and the expression profiles of D6, it has commonly been grouped together with DARC, another promiscuous receptor for chemokines. The Duffy antigen is the erythroid chemokine receptor (DARC) for the malaria-causing protozoan *Plasmodium vivax* and a highly promiscuous receptor that has the unique capacity to bind members of both CXC and CC branches. Despite having a seven-transmembrane domain architecture, the binding of ligands to Duffy antigen/receptor for chemokines (DARC) does not induce signal transduction and or appear to be coupled to a G-protein (Hadley and Peiper, 1997). Therefore, the precise physiological role of DARC has yet to be elucidated.

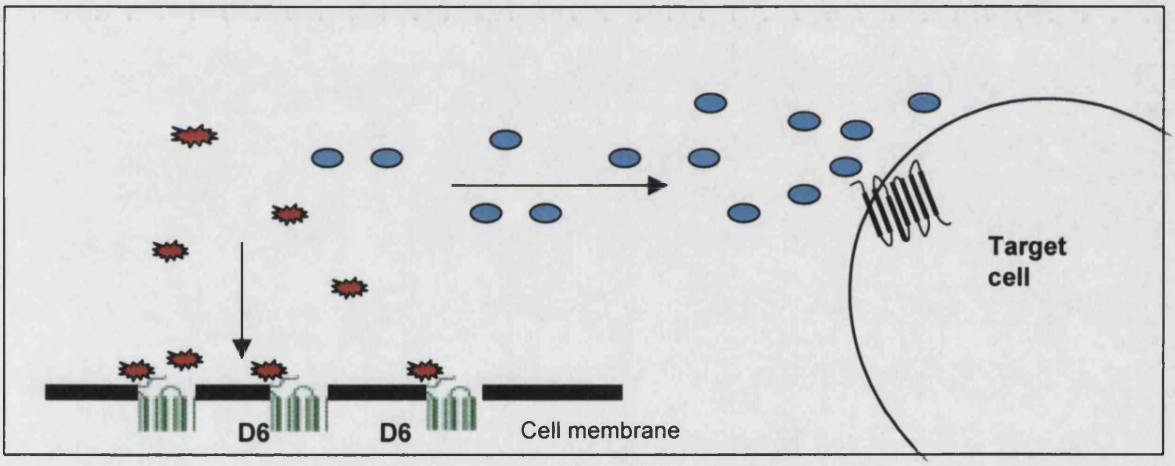
Some of our hypothesised functions of D6 are reminiscent of the suggested roles of the DARC receptor. Firstly, it is possible that D6 may act as a "sink" for chemokines in which they may be impounded and neutralised. Here, pro-inflammatory D6 chemokines (which happen to be the majority of it ligands) would be seized, but other non-D6 binding chemokines likely to be involved in constitutive leukocyte trafficking would be liberated to interact with target cells. This anti-adhesion mechanism may also function to inhibit chemokine-mediated firm adhesion of leukocytes in systems, such as the lymphatics, whereby a passive flow of cell populations is required.

Alternatively, the chemokine may in fact remain fully functional on binding to D6, but once bound would be presented by the receptor to leukocytes (the chemokine presenting molecule theory"). Typically, tissue-derived chemokines actuate inflammatory leukocyte emigration by traversing the endothelial cells (ECs) by means of chemokine diffusion through the intercellular gaps. However, Middleton *et al*,

demonstrated by electron microscopy that the IL-8 can be internalised by venular ECs, transcytosed to the luminal surface and presented to adherent leukocytes on the EC membrane. Thus, chemokine presentation could offer another functional role for the promiscuous D6 receptor.

Although such theories have so far offered plausible explanations, the emergence of signalling data from our studies are leading us away from the idea that D6 is a DARC-like receptor, and towards an entirely new perspective. Further *in vivo* and *in vitro* approaches are required to determine which hypothesis best reflects the function of D6 on lymphatic EC's, vascular tumours, and leukocytes.

Figure 7.6. The "chemokine sink theory". Pro-inflammatory CC chemokines that bind to D6 are sequestered and rendered inactive by the receptor. Non-D6 bindingchemokines are therefore lierated to interact with target cells and mediate constitutive trafficking of leukocytes



D6 – A model for generic CC chemokine receptors

The characterisation of D6 cDNA. Although D6 contains the predicted seven-transmembrane spanning regions, with residues and phosphorylation sites common to CC chemokine receptors, there are subtle differences in primary structure that may help to explain its character. As previously discussed, the conserved DRY motif

which is involved in G protein docking is altered to DKY in D6. Such a change would be assumed to affect the selection of G protein partners, however, the observed coupling of D6 transfectants lacking the DRY motif to PI3Kγ suggests that this motif may not be prerequisite for G protein interaction. The DKY-DRY mutant appeared to generate phosphatidic acid (PA) upon stimulation with MIP-1α, albeit with less efficacy than the wild-type. This was a surprising result since one would expect the restoration of the common DRY motif would potentiate the signalling capacity. Such a finding has prompted us to question the importance of this motif in the general signal transduction of CC chemokine receptors.

The N at position 92 in D6 is D in all other receptors (except DARC), and, if this D is mutated to N in CCR5, then the receptor (like D6) is no longer able flux calcium following ligand binding (R. Nibbs, personal communication). Coupling to the PI3Kγ was observed in the N92D mutant upon MIP-1α-stimulation that was considerably more potent than its wild-type counterparts. It was apparent that the N92D mutant was far more effective in coupling to the GPCR-specific PI3K, and thus, provides evidence that the D residue at position 92 in CC receptors is key to effective signal transduction.

Promiscuity

So why is D6 so promiscuous? It is possible that the duplicity of the D6 ligands allows differential regulation of receptor activation by different stimuli or tissue locations. This would give rise to a stimulus-dependent intensity, duration, or tissue-distribution of chemokines that recruit D6-bearing chemokines. Promiscuity of receptors allows its cognate ligands to have freedom for selectivity of the cellular response to alterations in environmental stimuli and conditions. It also bears relevance in the pathogenesis of a number of chemokine-mediated disease states. For example, different HIV strains possess some degree of chemokine-receptor promiscuity as the virus searches for $CD4^+$ chemokine-receptor positive cell (Xiao *et al*, 1998). Similarly, chronic inflammatory disorders, including allergy have also shown to be modulated by multiple chemokine receptors and receptors. Conditions of airway hyperresponsiveness, such as asthma, appear to be operated by a similar chemokine-chemokine receptor system, whereby the CCR3 receptor is expressed on a number of cells involved in allergy, such as mast cells, basophils and Th2 lymphocytes. The link between these cells and the pathogenesis of allergic inflammation has encouraged intense efforts to determine the link between the CCR3

receptor and its ligands, however these efforts are complicated by their promiscuity in receptor usage (Lilly and Daugherty, 2001).

With the recent demonstration of D6 expression on vascular tumour cells, it is possible that this receptor could have a significant role in tumourigenesis. In this regard, the D6-chemokine interaction could affect the leukocyte infiltrate, or the overall response of the tumours to inflammatory mediators. Further investigation into the biochemical and functional capacity of this receptor is required to demonstrate how D6 acts upon the biology of tumours and cell migration.

Final discussion

In the sub-section discussions, we have addressed the scientific issues facing this work. Here, in our final comment, we have opened a forum for the consideration of the general issues arising from the project. We discuss the validity of our experimental model, the clinical application of our findings, and the outlook for the future.

Experimental Model

In this study, we used the leukaemic monocytic cell line, THP-1, due to its characteristic endogenous expression of CCR2. As previously discussed, we were confident that this cell line was a robust model in which to carry out biochemical and functional investigations, mainly because other workers had achieved reliable signalling data from these cells. Although allowing detailed manipulation of the regulatory pathways involved, the system was not sufficient to enable the pursuit of the physiological issues concerning CCR2, i.e. it's pro-inflammatory role in chronic disease. That said, using the technology and the resources available, the combination of biochemical and functional studies described herein provided an appropriate *in vitro* model of chemokine-modulated cell migration. With regards to analysing signalling pathways, there were a number of minor limitations in using THP-1 cells. Firstly, there could, on occasion, be inconsistencies in the basal signalling activity of the cells, thus rendering some of the data as invalid. This was not the first report of such inconsistencies in monocytic cell lines, and in many cases, it has been ascribed to the 'mechanical stretch' that the cells are exposed to during the stimulation process (see Section 3- *Results and Discussion*).

Secondly, due to the observed mRNA expression of CCR1 and CCR3 (alternative receptors for MCP ligands), we had to ensure that all observed outputs were due to CCR2 engagement. Fortunately, this was achieved in full with the use of CCR2 antagonists. Another option would have been the use of CCR2 transfectants, such the 'CCR2b-HEK-283' employed by Turner *et al* and Berkhout *et al*. This model has been particularly useful in that it has highlighted specific regions of the receptor important for functions, and has demonstrated the differential regulation by the two CCR2 splice variants. However, as attractive as the HEK-293 model may seem, the somewhat 'abrasive' process of introducing genetic material into intact cells could induce other non-specific phenotypic changes in this system. To further validate the physiological relevance of our findings, it may have been useful to have conducted

some representative studies in human monocytes, and perhaps compare normal monocytes with those derived from atherosclerotic lesions. However, given the inter-donor differences observed in such studies, this may not have proved that useful given the constraints of time and the original objectives that we set out to achieve.

It is fair to say that the chemotaxis assays used in this thesis are the most suitable to the physiological conditions that we were attempting to simulate. The method, which is based on 'Boyden's chamber', has been the most widely used system in the context of chemokine biology. It allows the quantitative measurement of cell migration towards a chemoattractant, and permits the comparison of concentration gradients and at least two separate experimental conditions (e.g. plus/minus pertussis toxin, MCP-1 vs MCP-2). The principle drawback of using such a system is in that it does not take into consideration of the various transendothelial interactions that have been shown to be so crucial to the chemotactic process. However, we are confident that our assays were the most appropriate and representative way of analysing cell migration in the *in vitro.*

MCP-1 vs. MCP-2, MCP-3 and MCP-4

As highlighted in each section, there are many elements of regulation involved in CCR2 signalling pathways. Due to the range of pathways investigated (MAPK, SAPK, PLD, GTPases, and PI3K) in response to the four distinct CCR2 ligands (MCP-1, -2, -3 and –4) it would be unwise to propose a comprehensive working model for the CCR2 signalling system. This project has been a useful exercise in establishing general signalling events, but perhaps in retrospect it may have been more useful to concentrate on the events elicited by one particular pathway or CCR2 ligand. Our primary aim was not to compare the behaviour or efficacy of each MCP chemokine, but rather to verify that they were all capable of inducing signal transduction in a comparable manner to that observed with MCP-1. This was achieved on a number of levels, and in fact we were able to observe slight variations in behaviour and efficacy between the four. In terms of physiological relevance, these observations could be applied to an environment whereby CCR2 is exposed to all four ligands (e.g. the atheroslerotic lesion or rheumatic joint). The differences in binding affinity, signalling capacity and kinetics could be a strategic mechanism through which to stagger the biochemical events triggered by the receptor – and perhaps permit the strategic 'firing' of the signalling components and subsequent functional responses. We did not fully investigate MCP-2, -3 and –4 signalling

profiles enough to further subscribe to this hypothesis, although it does offer interesting concept

Application to the pathophysiology of atherosclerosis

Throughout this thesis there has been little reference to the pathophysiological or therapeutic applications of our findings in terms of the chronic inflammatory disorders. This is mainly due to the fact that the model, although relevant, is too far removed from the complexities of the arterial and vascular microenvironment. However, it is from investigations such as this that large-scale comprehensive research programmes arise. The application of *in vitro* data to whole organ systems has previously yielded invaluable information with regards to the pathology of disease and can provide a working model in the search for therapeutic targets.

In general therapeutic terms – there are two possible areas of clinical focus that have arisen through our investigations: 1) targeting he interaction of chemokines with CCR2, and; 2) manipulating the regulation of cell function by PI3K.

1) This refers to the simple observation that MCP chemokines actively induced the migration of inflammatory cells. It is no surprise that obstruction of this association by therapeutic means could attenuate cell migration and subsequently reduce inflammation. This concept has been implemented in a wide range of contexts, (including that of CCR2) in the form of receptor antagonists. These have shown to be very effective therapeutic tools but only when the antagonists are effectively localised to the desired area. For instance, in the atherosclerotic patient, inhibition of the CCR2-chemokine interaction in its entirety would indeed reduce the accumulation of macrophages in the cholesterolaemic artery, but it would also simultaneously inhibit systemic macrophage function, and consequently create a platform for immunodeficiency. In recent years, the implementation of elegant recepterology has permitted the identification of the precise residues necessary for chemokine signal transduction. This, in hand with more sophisticated methods of localising drug action, will eventually pave the way for more selective anti-chemokine therapy.

2) The most prominent feature of our findings is the impact of PI3K on CCR2-mediated signal transduction and cell migration. The further observation of differential regulation of PI3K by CCR2 makes this enzyme an ideal candidate for therapeutic targeting. Although our studies were not extensive enough to demonstrate the functional specialisation of the PI3K isoforms, we were able to

dissect out which of these were activated, their kinetic profile, and their contribution to D-3-lipid accumulation. This biochemical data provides a clear framework from which further functional investigations could develop. We have shown here that each MCP-1 activates a least three separate PI3K isoforms, and that broad-spectrum pharmacological inhibition blocks cell migration. Future studies will be aimed at making the link between biochemistry and functional specialisation.

PI3K: An appropriate target for the therapy of immunological disorders?

As the evidence implicating their involvement in disease processes accumulates, the PI3Ks appear to be increasingly attractive targets for the treatment of inflammatory and immunological disorders, not to mention cancer and other proliferative conditions. The elucidation of the functional specialisation of the individual PI3K isoforms will enable us to selectively inhibit them with an acceptable level of toxicity. It stands to reason here modulation of PI3K may prove useful in the treatment of atherosclerosis, as well as other vascular disorders such as re-stenosis following balloon angioplasty, diabetic retinopathy and fibrosing alveolitis.

Since PI3K is a ubiquitous enzyme family that regulates a diverse (and often contrasting) range of cellular processes, chronic inhibition of this pathway is likely to yield undesirable results. It may be more beneficial to look further into these signalling cascades and perhaps concentrate on effectors downstream of the individual isoforms, thus developing more stringency in the targeting of pathways specific functional responses.

D6.

As a subsidary to this project, we also investigated the signalling potential of the promiscuous CC chemokine receptor D6. We present here the first report of any signalling events elicited through this receptor, and this has paved the way for some exciting future studies. What makes these investgations more interesting is that D6 could well have a function quite unique from those normally associated from chemokine receptors. Elegant studies are currently being considered in our laboratory to extend this research. Moreover, the process of elucidating the novelties of D6 receptor will at the same time help us to understand the signalling behaviours of CC chemokine receptors in general.

Specific guidelines for future studies are outlined at the end of Sections 3,4,5,6 and 7.

References

Alblas J, van Corven A, Hordijk P, Milligan P, Moolenaar W. (1993). G_i-mediated activation of the p21ras-mitogen-activated protein kinase pathway by alpha 2-adrenergic receptors expressed in fibroblasts . *J.Biol.Chem.* 268: 22235-22238

Alkhatib G, Ahuja SS, Light D, Mummidi S, Edward A, Berger A, Ahuja SK (1997). CC Chemokine Receptor 5-Mediated Signalling and HIV-1-Co-receptor Activity Share Common Structural Determinants. *J Biol. Chem.* 272:19771-19776.

Allen W, Zicha D, Ridley AJ, Jones GE. (1998) A role for Cdc42 in macrophage chemotaxis. *J. Cell. Biol.* 141:1147-1157.

Alsono A, Bayon Y, Renedo M, Sanchez Crespo M. (2000). Stimulation of FcγR Receptors Induces Monocyte Chemoattractant Protein-1 in the Monocytic Cell Line THP-1 by a Mechanism Involving IκB-α Degradation and Formation of p50/p65 NF-κB/Rel Complexes. *Int. Immunol.* 12:547-554.

Altman A, Mustelin T, Coggeshall KM. 1990. T lymphocyte activation: a biological model of signal transduction. *Crit. Rev. Immunol.* 10:347–91

Ando Y, Lazarus GS, Jensen PJ. (1993). Activation of PKC inhibits keratinocyte migration. *J Cell Physiol.* 156:487-496.

Aplin A, Howe A, Juliano R. (1999). Cell adhesion molecules, signal transduction and cell growth. *Curr. Opin. Cell. Biol.* 11-737-744.

Arai,H. Charo,I.(1996). Differential regulation of G-protein-mediated signaling by chemokine receptors *J. Biol. Chem.* 271, (36), 21814-21819.

Arcaro A, Zvelebil M, Wallasch, Ullrich A, Waterfield M, Domin J. (2001). Class II Phosphoinositide 3-kinases are downstream targets of activated polypeptide growth factor receptors. *Mol. Cell.Biol.* 20(11):3817-3830.

Ashida N, Arai H, Yamasaki M, Kita T. (2001). Distinct signalling pathways for MCP-1-dependent Integrin activation and chemotaxis. *J. Biol. Chem.* 276:16555-16560.

Baggiolini M, Dewald B, Schnyder J, Ruch W, Cooper PH, Payned TG. (1987). Inhibition of the phagocytosis-induced respiratory burst by the fungal metabolite wortmannin and some analogues. *Experimental Cell Research.* 169:408-418.

Baggiolini M, Dewald B, Moser B. (1997). Human Chemokines: An Update. *Ann. Rev. Immunol.* 15:675-705.

Baggiolini M. (1998). Chemokines and leukocyte traffic. *Nature.* 392:565-568.

Bacon K, Premack B, Gardner P, Schall TJ. (1995). Activation of dual T cell signalling pathways by the chemokine RANTES. *Science.* 269:1727-1729

Bacon, K. Schall, T. Dairaghi, D (1998). RANTES Activation of Phospholipase D in Jurkat T Cells: Requirement of GTP-Binding Proteins ARF and RhoA. *J. Immunol.* 160, 1894-1900.

Balendran A, Casamayor A, Deak M, Paterson A, Gaffney P, Currie R. (1999). PDK1 aquires PDK2 activity in the presence of a synthetic peptide derived from the carboxy terminus of PRK2. *Curr. Biol.* 1999. 9:393:404.

Banno Y, Takuwa Y, Akao Y, Okamoto H, Osawa Y, Naganawa T, Nakashima S, Suh PG, Nozawa Y. (2001). Involvement of Phospholipase D in Sphingosine 1-Phosphate-induced Activation of Phosphatidylinositol 3-Kinase and Akt in Chinese Hamster Ovary Cells Overexpressing EDG3. *J.Biol.Chem.* 276:35622-35628.

Benard V, Bohl B, Bokoch G. (1999). Characterisation of Rac and Cdc42 Activation in Chemoattractant-stimulated Human Neutrophils Using a Novel Assay for Active GTPases. *Journal of Biological Chemistry.* 274:13198-13204.

Berger, E and Dineter A. (1998). Golgi-disturbing Agents. *Histochem. Cell. Biol.* 109:571-590.

Berkhout, T. Saraus, H. (1997). Cloning *in Vitro* Expression and Functional Characterisation of a Novel Human CC chemokine of the Monocyte Chemotactic Protein (MCP) Family (MCP-4) That Binds and Signals through the CC Chemokine Receptor 2B. *J. Biol. Chem.* 272, (26), 1604-16413.

Berliner JA, Territo MC, Sevanian A, Ramin S, Kim A, Bamshad M, Esterson M, Fogelman A. (1990). Minimally Modified Low density Lipoprotein Stimulates Monocyte Endothelial Interactions. *J. Clin. Invest.* 85:1260.

Beron W, Alvarez-Dominguez D, Mayorga A, Stahl P (1995). Membrane trafficking along the phagocytic pathway. *Trends in Cell Biology.* 5:100-104

Berridge MJ. (1997) Elementary and global aspects of calcium signalling. *J.Physiol.* 499:291-306.

Bershadsky, A and Futerman, A. (1994). Distruption of the Golgi apparatus by Brefeldin-A blocks cell polarisation and inhibits directed cell migration. *PNAS.* 91:5686-5689.

Billah M, Anthes. (1990). The regulation and cellular function of phosphatidylinositolcholine. *Biochem.J.* 269:281.

Biondi R, Kieloch A, Currie R, Deak M, Alessi D. (2001). The PIF-binding pocket in PDK1 is essential for activation of S6K and SGK, but not PKB. *EMBO.* 20:4380-4390.

Blanpain C, Wittamer V, Vanderwinden J-M, Boom A, Renneboog B, Lee B, Le Poul E, Asmar L, Govaerts C, Doms R, Parmentier M. (2001) Palmitoylation of CCR5 is critical for receptor trafficking and efficient activation of intracellular signalling pathways. *J. Biol. Chem.* 276:23795-23804.

Blaukat A, Barac A, Cross M, Offermans S Dikic I. (2000). G protein-coupled receptor-mediated mitogen-activated protein kinase activation through cooperation of $G\alpha_q$ and $G\alpha_i$ signals. *Mol.Cell.Biol.* 20:6837-6848.

Bleul C, Fuhlbrigge R, Casanovas J, Aiuti A, Springer T. (1996). A Highly Efficacious Lymphocyte Chemoattractant, Stromal Cell-derived Factor-1 (SDF-1). *J. Exp. Med.* 184:1101-1109.

Bockaet J, Pin JP. (1999). Molecular tinkering of G-protein coupled receptor: an evolutionary success. *EMBO J.* 18:1723-1729.

Bondeva T, Pirola L, Bulgarelli-Leva G, Rubio I, Wetzker R, Wymann M. (1998). Bifurcation of Lipid and Protein Kinase Signals of PI3Kγ to the Protein Kinases PKB and MAPK. *Science.* 282, 293-296.

Bonnini JA. Martin S.K., Dralyuk F., Roe M.W., Philipson L.H.,RA Steiner D.F.; (1997). Cloning, expression, and chromosomal mapping of a novel human CC-chemokine receptor (CCR10) that displays high-affinity binding for RT MCP-1 and MCP-3.*DNA Cell Biol.* 16:1249-1256.

Bonnachi A, Romagnani P, Romanelli R, Efsen E, Annuziato F, Lasagni L, Francalanci M, Serios M, Laffi G, Pinzani M, Gentilini P, Marra F. (2001). Signal transduction by the chemokine receptor CXCR3. *J. Biol. Chem.* 276: 9945-9954.

Bonser RW, Thompson NT, Randall RW, Tateson JE, Spacey GD, Hodson HF, Garkand LG. (1993) Demethoxyviridin and wortmannin block phospholipiase C and D activation in the human neutrophil. *Brit. J.Pharm.* 103:1237-1241.

Boring, L. Gosling, J . Cleary M, Charo IF. (1998). Decreased lesion formation in CCR2-/- mice reveals a role for chemokines in the initiation of atherosclerosis. *Nature.* 394, 894-97

Bousiotis VA, Freeman GJ, Berezoyskaya A, Barber DL, Nadler LM. (1997). Maintenance of human T cell anergy: blocking of IL-2 gene transcription by activated Rap1. *Science.* 278(5335):124-8.

Brown RA, Domin J, Acaro, A, Waterfield MD, Sheperd PR (1999). Insulin Activates the Isoform of Class II Phosphoinositide 3-Kinase *J.Biol.Chem.* 274, 14529-14532.

Brunet A, Bonni A, Zigmond MJ, Lin MZ, Lin P, Jou LS, Hu M, Anderson, Arden K, Blenis J, Greenberg. (1999). Akt promote cell survival by phosphorylating and inhibiting a forkhead transcription factor. *Cell.* 96:857-868.

Burgering BM and Coffer PJ. (1995). Protein kinase B (c-Akt) in phosphatidylinositol-3-OH kinase signal transduction. N*ature.* 376:599-602.

Butcher EC, Picker LJ. (1996). Lymphocyte homing and homeostasis. *Science.* 272:60.

Campbell J, Pan J, Butcher E. (1999). Developmental switches in chemokine responses during T cell maturation. *J. Immunol.* 2353-2357.

Cambien B, Pomeranz M, Millet MA, Rossi B, Schmid-Alliana A. (2001). Signal transduction involevd in MCP-1-mediated transendothelial migration. *Blood.* 97 :359-366.

Cantley L, Neel, B. (1999). New insights into tumour suppression: PTEN suppresses tumour formation by restraining phosphoinositide 3-kinase/Akt pathways. *PNAS* (USA). 96: 4240-45.

Cerdan C, Devilard E, X Luc, Olive D (2001). The C-class chemokine lymphotactin costimulates the apoptosis of human $CD4^+$ T cells. *Blood.* 97: 2205-2212.

Charo, I. Myers, S. Herman, A. Franci, C. (1994). Molecular Cloning and Functional Expression of a Monocyte Chemotactic Protein Receptor. *PNAS.* 91, 2752-2756.

Cheever M, Sato T, De Beer T, Kutateladze T, Emr S, Overduin M. (2001) Phox domain interaction with PtdIns(3)P targets the Vam7 t-SNARE to vacuole membranes. *Nature Cell Biology.* 3:613-618.

Chen G, Hitomi M, Han J, Stacey D. (2000). The p38 Pathway Provided Negative Feedback for Ras Proliferative Signalling. *J.Biol.Chemistry.* 275:38973-38980.

Ching T, Wang D-S, Hsu A, Lu PJ, Chen C-S. (1999). Identification of multiple phosphoinositide-specific Phospholipases D as new regulatory enzymes for Phosphatidylinositol 3,4,5)-Trispshophate. *J. Biol. Chem.* 274:8611-8617.

Chou MM, Hou W, Johnson J, Graham LK, Lee MH, Chen -S, Newton Ac, Schaffhausen Toker A. (1998). Regulation of the PKC-ξ by the PI 3-kinase-PDK-1-Akt/PKB signalling pathway. *Curr. Biol.* 8:1069-1077.

Clapham D, Neer J. (1997). New roles for G protein βγ dimers in transmembrane signalling. *Nature.* 365:403-406.

Cocchi F, DeVico AL, Garzino-Demo A Arya SK, Gallo RC, Lusso P.(1995). Identification of RANTES, MIP-1 alpha, and MIP-1 beta as the major HIV-suppressive factors produced by CD8+ T cells. *Science.* 270:1811-1815.

Coffer PJ, Jin J, Woodgett J. (1998). PJ Coffer, J Jin, JR Woodgett. Protein kinase B (C-AKT) – a multifunctional mediator of phosphatidylinositol 3 kinase activation. *Biochemical Journal*, 335: 1-13.

Combadiere C, Ahuja SK, van Damme J. (1995).Monocyte chemoattractant protein-3 is a functional ligand for CC chemokine receptors 1 and 2B. *J Biol Chem.* 270:29671-29675.

Cook SJ, Wakelam M. (1992). Phospholipases C and D are involved in mitogenic signal transduction. *Rev. Physiol. Biochem.Pharmacol.* 119:13

Coso O, Chiariello M, Kalinec G, Kyriakis J, Woodgett J, Gutkind S. (1995). Transforming G Protein-coupled receptors potently activate JNK (SAPK). *J. Biol. Chem.* 270:5620-5624

Cox D, Tseng C, Bjekic G, Greenberg S. (1999). A requirement for phosphatidylinositol-3-kinase in pseudopod extension. *J.Biol.Chem.* 274:1240-1247.

Craddock B, Hobbs J, Edmead C, and Welham MJ. (2001) Phosphoinositide 3-Kinase-dependent Regulation of Interleukin-3-induced Proliferation. *J.Biol.Chem.* 276: 24274-24283.

Craddock B, Orchiston E, Hinton H, Welham M.. (1999) Dissociation of Apoptosis from Proliferation, Protein Kinase B Activation, and BAD Phosphorylation in Interleukin-3-mediated Phosphoinositide 3-Kinase Signaling. *J.Biol.Chem.* 274: 10633-10640.

Cross DAE, Allessi D, Cohen P, Andjekovich M, Hemmings BA. (1995). Inhibition of glycogen synthase kinase-3 by insulin-mediated protein kinase B. *Nature*. 378:785-789.

Cross DAE, Steward A, Hodgkin MN, Kerr DJ Wakelam MJO. (1995). Wortmannin and Its Structural Analogue Demethoxyviridin Inhibit Stimulated Phospholipase A_2 Activity in Swiss 3T3 Cells. *J.Biol.Chem.* 270: 25352-25355.

Cuevas B, Yiling Lu, Mao M, Zhang, LaPushin R, Siminovitch K, Mills G. (2001) Tyrosine Phosphorylation of p85 Relieves Its Inhibition Activity on Phosphatidylinositol 3-kinase. *J.Biol.Chem.* 276: 27455-27461.

Curnock A, Logan M, Ward S. (2002). Chemokine signalling: Pivoting around phosphoinositide 3-kinases. Immunology. 105(2):125-36. Review.

Dahinden C, Geiser T, Brunner T, von Tscharner V, Caput D, Ferrara P, Minty A, Bagiolini M. (1994). Monocyte chemotactic protein 3 is a most effective basophil- and eosinophil-activating chemokine *J. Exp. Med.* 179: 751-756

D'Ambrosio *et al*, (1998) Selective upregulation of chemokine receptors CCR4 and CCR8 upon activation of polarised human type 2 Ty cells. *J. Immunol.*161:5111-5115

De Camilli P, Emr SD, McPherson PS, Novick P. (1996). Phosphoinositides are regulators of membrane traffic. *Science.* 271:1533.

Della Rocca G, Biesen T, Daaka Y, Luttrell, Louis Luttrell, Lefkowitz R. (1997) Ras-dependent Mitogen-activated Protein kinase activation by G Protein-coupled receptors. *J.Biol.Chem.* 272:19125-19132.

Del Pozo M, Vincente-Manzanares M, Tejedor R, Serrador J, Sanchez-Madrid F. (1999). Rho GTPases control migration and polarisation of adhesion molecules and cytoskeletal ERM components in T lymphocytes. *Eur.J.Immunol.* 29:3609-20.

Dempsey E, Newton A, Mochly-Rosen D, Fields A, Reyland M, Insel P, Messing R. (2000). Protein kinase C isoenzymes and the regulation of diverse cell responses. *Am. J. Physiol. Lung. Cell. Mol. Physiol.* 279:L429-428

Dhand R, Hiles I, Panayotou G, Roche S, Fry MJ, Gout I, Totty NF, Thruong O, Vincendo P, Yonnezawa K, Kasuga M, Courtneidge SA, Waterfield M. (1994). PI 3-Kinase is a dual specificity enzyme:autoregulation by an intrinsic protein-serine kinase activity. *EMBO J.* 13: 522-533.

Di Christofano A, Pesce B, Cordon-Cardo C, Pandolfi P. (1998). PTEN is essential for embryonic develoment and tumour suppression. *Nat.Gen.* 19:348-355.

Dinter A, Berger E. (1998). Golgi-disturbing agents. *Histochem. Cell. Biol.* 109:571-590.

Domin J, Waterfield M. (1997). Cloning of a phopshoinositol 3-kinase with a C2 domain that displays reduced sensitivity to the inhibitor wortmannin. *Biochem.J.* 326: 139-147.

Domin J, Gadarov I, Smith M, Kee JH, Waterfield MD. (2000). The class II PI3K-C2α is concentrated in the trans-Golgi network and is present in clathrin-coated vesicles. *J.Biol.Chem.* 275:11943-50

Dominguez I, Itoh K, Sokol SY. (1995). Role of glycogen synthase kinase 3 beta as a negative regulator of dorsoventral axis formation in *Xenopus* embryos. *PNAS.* 92:8498.

Downward J. (1998) Ras signalling and apoptosis. *Curr.Opin.Gen.Dev.* 8:49-54.

Egashira K, Koyanagi M, Kitamoto S, Ni W, Kataoka C, Morishita R, Kaned Y, Akiyama C, Nishida K, Sueishi K, Takeshita A. (2001). Anti-monocyte chemoattractant protein-1 gene therapy inhibits vascular remodelling in rats: blockade of MCP-1 activity after intramuscular transfer of a mutant gene inhibits vascular remodelling induced by chronic blockade of NO synthesis. *FASEB.* 14: 1974-1978.

Emoto, M., Klarlind, J. K., Waters, S. B., Hu, V., Buxton, J. M., Chawla, A., and Czech, M. P. (2000) A role for phospholipase D in GLUT4 glucose transporter translocation*J. Biol. Chem.* 275, 7144-7151.

English D. (1996). Phosphatidic acid: A lipid messenger involved in intracellular signalling. *Cell. Signal.* 341-347.

Epand R, Stafford AR. (1990). Counter-regulatory effects of phosphatidic acid on protein kinase C activity in the presence of calcium and diolein.. *Biochem.Biophys.Res.Comm.* 171, 487-490.

Exton J. (1998). Small GTPases Minireview series. *J. Biol. Chem.* 272:19923-20688

Exton, JH. (1999). Regulation of phospholipase D. *Biochimica et Biophysica Acta.* 1439, 121-133.

Fang X, Yu S, Lu Y, Bast R, Jr., Woodgett J, Mills G. (2000). Phosphorylation and inactivation of glycogen synthase kinase 3 by protein kinase *PNAS*, 97: 11960 11965

Fantuzzi, L. Borghi, P. Ciolli, V. Pavlakis G, Belardelli F, Gessani S (1999). Loss of CCR2 expression and function response to Monocyte chemoattractant protein (MCP-1) during differentiation of human monocytes: Role of secreted MCP-1 in the regulation of the chemotactic response. *Blood.* 94, (3), 875-883.

Faure, M. Voyno-Yasenetskya, T (1999). cAMP and beta gamma subunits of heterotrimeric G proteins stimulate the mitogen-activated protein kinase pathway in COS-7 cells *J.Biol.Chem.* 269, 7851-7854

Feniger-Barish R, Ran M, Zaslaver A, Ben-Baruch A. (1999). Differential modes of regulation of cxc chemokine-induced internalization and recycling of human CXCR1 and CXCR2. Cytokine. 1999;11:996-1009

Fine J, Byrnes H, Zavodny P, Hipkin W. (2001). Evaluation of Signal Transduction Pathways in Chemoattractant-Induced Human Monocyte Chemotaxis. *Inflammation.* 25:61-66

Fevert E, BjorbaeK, Veneble C, Keller S, Kahn B. (1998). Targeting of Constitutively active Phosphoinositide 3-Kinase to GLUT4-containing Vesicles in 3T3-L1 Adipocytes. *J.Biol.Chem.* 273: 25480-25487.

Frohman M, Morris AJ. (1996). Mammalian phospholipase D structure and regulation.*Curr. Biol.* 6:945.

Frohman, M. Sung, T. Morris, A. (1999). Endosomal localization of phospholipase D 1a and 1b is defined by the C-termini of the proteins, and is independent of activity *Biochimica et Biophysica Acta*. 1439, 175-186

Fruman D, Snapper S, Yballe, davidson L, Yu J, Alt, Cantley L. (1999). Impaired B cell development and proliferation in the absence of phosphoinositide 3-kinase p85α. *Science*. 283: 393-397.

Gahmber C, Valmu L, Kotovuori A, Kotovuori P, Hilden T, Fagerholm S, Kantor C, Nurminen T, Ihanus E, Tian L. (1999) Leukocyte Adhesion – an Integrated molecular Process at the Leukocyte Plasma Membrane. *Biosci. Reports.* 19:273-279.

Ganju RK, Brubaker SA, Meyer J, Dutt P, Yang Y, Qin S, Newman W, Groopman JE. (1998) The α-chemokine, stromal cell-derived factor-1α, binds to the transmembrane G-protein-coupled CXCR-4 receptor and activates multiple signal transduction pathways. *J. Biol. Chem.* 273:23169-23175.

Gangarosa LM, Seizmore N, Graves-Deal R, Oldham SM, Der CJ, Coffey AJ. (1997) A Raf-independent Epidermal Growth Factor Receptor Autocrine Loop Is Necessary for Ras Transformation of Rat Intestinal Epithelial Cells. *J.Biol.Chem.* 272:18926-18931

Gerard C and Rollins B. (2001) Chemokine and Disease. *Nature Immunol.* 2:108-115.

Gharaee-Kermani M, Denholm EM, Phan SH. (1996). Costimulation of Fibroblast Collagen and Transforming Growth Factor-β1 Gene Expression by Monocye Chemotactic Protein-1 via Specific Receptors. *J. Biol. Chem.* 271:17779:17784.

Goebler M, Gillitzer R, Kilian K, Utzel K, Brocker EB, Rapp U, Ludwig S. (2001). Multiple Signaling Pathways regulate NF-κB-Dependent Transcription of the Monocyte Chemoattractant Protein-1 Gene in Primary Endothelial Cells. *Blood.* 97:46-55.

Gong, X. Gong, W. Kuhns, D. (1997). Monocyte Chemotactic Protein-2 Activates CCR5 and Blocks CD4/CCR5-mediated HIV Entry/Replication. *J.Biol. Chem.*. 272, (18), 11682-5.

Gonzalo J, *et al*, (1998). The co-ordinated action of CC chemokines in the lung orchestrates allergic inflammation and airway hyperresponsiveness. *J. Exp. Med.* 188:157-167.

Gosling J. Slaymaker S, Gu L. (1999) MCP-1-deficiency reduces susceptibility to atherosclerosis in mice that overexpress apolipoprotein B. *J Clin Invest.* 103:773

Grange M, Sette C, Cuomo M, Conti M, Lagarde M, Prigent AF, Nemoz G. (2000). The cAMP-specific Phosphodiesterase PDE4D3 is regulated by phosphatidic acid binding. *J. Biol. Chem.* 275:33379-33387.

Gu L, Okada Y, Clinton SK. (1998). Absence of monocyte chemoattractant protein-1 reduces atherosclerosis in low density lipoprotein receptor-deficient mice. *Mol. Cell.* 2:275.

Gutkind JS. (1998). The Pathways Connecting G Protein-coupled Receptors to the Nucleus through Divergent Mitogen-activated Protein Kinase Cascades *J. Biol. Chem.* 273: 1839-1842

Gratton JP, Morales-Ruiz M, Kureishi Y, Fulton D, Walsh K, Sessa WC. Akt down-regulation of p38 signaling provides a novel mechanism of vascular endothelial growth factor-mediated cytoprotection in endothelial cells.*J Biol Chem.* 276(32):30359-65.

Grewal IS, Rutledge BJ, Fiorillo JA, Gu L, Gladue RP, Flavell RA, Rollins BJ: Transgenic monocyte chemoattractant protein-1 (MCP-1) in pancreatic islets produces monocyte-rich insulitis without diabetes: Abrogation by a second transgene expressing systemic MCP-1, *J Immunol* 159:401 -408, 1997

Grynkiewicz G, Poeniw M Tsien Y. (1985). A new generation of calcium indicators with greatly improved fluorescence properties. *J.Biol.Chem.* 260:3440-3450.

Haddad E, Zugaza J, Louache F, Debili N, Crouin C, Schwarz K, Fischer A, Vainchenker W, Bertoglio J. (2001). The Interaction between Cdc42 and WASP is required for SDF-1-induced T-Lymphocyte chemotaxis. *Blood.* 97(1):33-37

Hadley, T.J., and S.C. Peiper. (1997). From malaria to chemokine receptor: The emerging physiological role of the Duffy blood group antigen. *Blood* 89:3077-3091.

Hall, A. (1998) Rho GTPases and the Actin Cytoskeleton. *Science.* 279:509-514.

Hamm, H. E. (1998). The many faces of G protein signaling. *J. Biol. Chem.* 273: 669-672.

Hammond S, Jenco J, Nakashima S Cadwallader K, Gu Q, Cook S, Nozawa Y, Prestwich G, Frohman M, Morris A. (1997) Characterization of Two Alternately Spliced Forms of Phospholipase D1. Activation of the purified enzymes by phosphatidylinositol 4,5-bisphosphate, adp-ribosylation factor, and rho family monomeric gtp-binding proteins and protein kinase c-α *J. Biol.Chem.* 272:3860-3868.

Hammond, S. Altshuller Y, Sung TC, Rudge S, Rose K, Engebrecht J, Morris AJ, Frohman M. (1999). Human ADP-ribosylation Factor-activated Phosphatidylcholine-specific Phospholipase D Defines a New and Highly Conserved Gene Family *J.Biol.Chem.* 270, 29640-

Han K, Tangirala R, Green S, Quehenberger O. (1998). Chemokine Receptor CCR2 expression and Monocyte Chemoattractant protein-1-mediated chemotaxis in human monocytes. *Atheroscler. Thromb. Vasc. Biol.* 18:1983-1991.

Hancock WW, Gao W, Faia KL, Csizmadia V. (2000). Chemokines and their receptors in allograft rejection. *Curr. Opin. Immunol.* 511-516.

Hara, K Yonezawa, H Sakaue, A Ando, K Kotani, T Kitamura, Y Kitamura, H Ueda, L Stephens, TR Jackson, MD Waterfield, and M Kasuga. (1994) 1-Phosphatidylinositol 3-Kinase Activity is Required for Insulin-Stimulated Glucose Transport But not for RAS Activation in CHO Cells. PNAS 91: 7415-7419

Haribabu B, Zhelev D, Pridgen B, Richardson R, Ali H, Synderman R. (1999). Chemoattractant Receptors activate distinct pathways for chemotaxis and secretion. *J. Biol. Chem.* 274:37087-37092.

Harper S, LoGrasso P. (2001). Signalling for survival and death in neurones. The role of stress-activated kinases, JNK and p38. *Cellular Signalling.* 13:299-310.

Hart AC, Kramer H, Van Vactor DL, Paidhungat M, Zipursky SL. (1990). Induction of cell fate in the Drosophila retina: The bride of sevenless protein is predicted to contain a large extracellular domain and seven transmembrane segments. *Genes Dev* 4:1835.

Harwood AJ, Plyte SE, Woodgett J., Strutt H, Kay RR. (1995). Glycogen synthase kinase 3 regulates cell fate in Dictyostelium. *Cell.* 80:139-148

Haslam RJ, Koide HB, Hemming BA. (1993). Plecktrin domain homology. *Nature* 363:309-310.

Hawes. BE, Luttrell LM, VanBiesen T, Lefkowitz RJ. (1996) Phosphatidylinositol 3-kinase is an early intermediate in the G$\beta\gamma$-mediated mitogen-activated protein kinase signalling pathway. *J. Biol. Chem.* 271: 12133-12136.

Hendrick, J.A, and A Zlotnik (1996). *Current Opinions in Immunology.* 8, 343.

Heldin, K. Bell, M. Huntoon, C. (1999). G_i Proteins use a novel PKC-ζ and Ras-independent pathway to activate extracellular signal-regulated kinase and mobilise AP-1 transcription factors in Jurkat T lymphocytes. *J.Biol.Chem.* 274 (28), 19992-2001.

Heldin C, Purton M. (1996). Signal Transduction. Ed. Carl-Heldin and Mary Purton. Published by Chapman & Hall.

Helwig J, Bockock G, Carpenter C, Jammey P, Taylor A, Toker A. (1995). Thrombin receptor ligation and activated rac uncap actin filament barbed ends through phosphoinositide synthesis in permeabilised human platelets. *Cell.* 82:643-653.

Helwig, U, Lammers, K M, Gionchetti, P, Rizzello, F, Campieri, M, Uguccioni;, M, Wedemeyer, J. Lorentz, A, Manns, M P, Bischoff, S C. (2000). MCP-3 in inflammatory bowel disease (reply) *Gut.* 47:155-155.

Hershberg RM, Mayer LF.(2000). Antigen processing and presentation by intestinal epithelial cells - polarity and complexity. Immunol Today 2000 Mar;21(3):123-8

Hesselgesser J, Liang M, Hoxie J, Greenberg M, Brass LF, Orsini MJ, Taub D, Koruk R. (1998). Identification and characterisation of the CXCR4 chemokine receptor in human T cell lines: Ligand binding, biological activity and HIV-1 infectivity. *J. Immunol.* 160:877-883.

Hessen M, Tanabe S, Berman SA, Yoshizawa I, Luo Y, Kim RJ, Post TW. (1996) Mouse Astrocytes Respond to the Chemokines MCP-1 and KC, but reverse transcriptase-polymerase chain reaction does not detect mRNA for the KC or the new MCP-1 receptor. *J. Neurosci. Res.* 45:382-391.

Hibi M, Lin a, Smeal T *et al.* (1993) Identification of an onco-protein and UV-responsive protein kinase that binds and potentiates the c-Jun activation domain. *Gen.Dev.* 7:2135-48.

Hirsh E, Kantanaev VL, Garlanda C, Azzolino O, Pirola L, Silengo L, Sozzani S, Mantovani A, Altruda F, Wymann MP. (2000) Central role for G protein-coupled phosphoinositide 3-kinase-γ in inflammation. *Science.* 287:1049-1053.

Hmama Z, Herrera Veli P, Nandan D, Reiner NE. Monocyte adherence induced by lipopolysaccharide involves CD14, LFA-1 and cytohesin-1: regulation by Rho and phophatidylinositol 3-kinase. *J. Biol. Chem.* 274:1050-1057.

Hoffbrand A, Pettit J. (1992). Essential Haematology. 3rd Edition. Blackwell Sciences.

Hoogewerf, A. Kuschert G, Proudfoot A, Borlat A, Clark-Lewis D, Power C, Wells T. (1997). Glycosaminoglycans mediate cell surface oligomerization of chemokines. *Biochemistry 36:13570.*

Hordijk, L, Verlaan, Van Corven E, Moolenaar W. (1994). Protein tyrosine phosphorylation induced by lysophosphatidic acid in Rat-1 fibroblasts. Evidence that phosphorylation of map kinase is mediated by the Gi-p21ras pathway. *J.Biol Chem.* 269:645-651.

Itoh H, Yamamura S, Ware A, Zhuang S, Mii S, Liu B, Kent C. (2001). Differential effects of protein kinase C on human vascular smooth muscle cell proliferation and migration. *Am.J.Physiol.Heart.Circ.Physiol.* 281 :H359-H370.

Iglesias, T. Waldron, R. (19998). *Journal of Biological Chemistry.* 273, 27662-27667.

Jahnsen F, Haye R, Gran E, Brandtzaeg P, Johansen F-E. (1999). Glucocorticosteroids Inhibit mRNA Expression for Eotaxin, Eotaxin-2, and Monocyte-Chemotactic Protein-4 in Human Airway Inflammation with Eosinophilia. *J.Immunol..* 163: 1545-1551.

Jiang, H., Luo, J.-Q., Urano, T., Frankel, P., Lu, Z., Foster, D.A., and Feig, L.A. (1995). Involvement of Ral GTPase in v-Src-induced phospholipase D activation. *Nature* 378, 409-412.

Jimenez C, Portella R, Mellado M, Rodriguez-Frade J, Collard J, Serrano A, Martinez A, Avila J, Carrera C. (2000). The role of the PI3K regulatory subunit in the control of actin organisation and cell migration. *J. Biol. Chem.* 151:249-262.

Jin T, Zhang N, Long N, Parent C, Devreotes P. (2000). Localisation of the G protein βγ complex in living cells during chemotaxis. *Science*, 287:1034-1036.

Jing Q, Xin SM, Zhang WB, Wang P, Qin YW, Pei G. (2000) Lysophosphatidylcholine activates p38 and p42/44 mitogen-activated protein kinases

in monocytic THP-1 cells, but only p38 activation is involved in its stimulated chemotaxis. *Circ. Res.* 87: 52-59.

Jones DT and Reed RR. (1987) Molecular cloning of five GTP-binding protein cDNA species from rat olfactory neuroepithelium. *J. Biol. Chem.* 272: 14241-14249

Jones SA, Moser B, Thelen MA. (1995). Comparison of post-receptor signal transduction events in Jurkat cells transfected with either IL-8R1 or IL-8R2: Chemokine-mediated activation of p42/p44 MAPK (ERK2). *FEBS Lett.* 364:211-214.

Juffermans N, Dekkers P, Peppelenbosch M,Speelman P,. Van Deventer, Van der Poll T. Expression of the Chemokine Receptors CXCR1 and CXCR2 on Granulocytes in Human Endotoxemia and Tuberculosis: Involvement of the p38 MitogenActivated Protein Kinase Pathway.

Just II, Selzer, Wilm M, von Eichel-Streiber C, Mann M, Aktories K. (1995) Glucosylation of Rho proteins by *Clostridium difficile* toxin B. *Nature* 375, 500-503. Kaneki (1999). JNK Myeloid

allergy. *Int. Arch. Allergy. Imm.* 124:423-431.

Karnam P, Standaert M, Galloway L, Farese R. (1997). Activation and Translocation of Rho (and ADP Ribosylation Factor) by Insulin in Rat Adipocytes. APPARENT IVOLVEMENT OF PHOSPHATIDYLINOSITOL 3-KINASE *J. Biol. Chem.* 1997 272: 6136-6140

Katan M (1998). Famillies of phosphoinositide-specific phospholipase C: Structure and function. *Biochem.Biophys.Acta.* 1436:5-17.

Kitayama J, Carr MW, Roth SJ, Buccola J, Springer TA. (1997). Contrasting responses to multiple chemotactic stimuli and transendothelial migration: heterologous desensitisation in neutrophils and augmentation of migration in eosinophils. *J. Immunol.* 158:2340.

Kim C, Hangoc G, Cooper S, Helgason C, Yew S, Humphries RK, Krystal G, Broxmeyer H. (1999). Altered responsiveness to chemokines due to targeted disruption of SHIP. *J. Clin. Invest.* 12:104.

Klein PS, Sun TJ, Saxe CL, Kimmel AR, Johnson RL, Devreotes PN (1998). A chemoattractant receptor controls development in Dictyostelium discoideum. *Science* 241:1467

Knall C., Worthen G.S. and Johnson G.L.(1997) Interleukin 8-stimulated phosphatidylinositol-3-kinase activity regulates the migration of human neutrophils independent of extracellular signal-regulated kinase and p38 mitogen-activated protein kinases. *Proc.Natl.Acad.Sci. U. S. A*, 3052-3057.

Ktistakis N, Brown H, Sternweis P, Roth M. (1995). Phospholipase D is present in Golgi-enriched membranes and its activation by ADP-ribosylation factor is sensitive to Brefeldin A.*PNAS.* 92:4952-

Kyriakis JM, Banerjee P, Nikolakaki E, Dai T, Rubie EA, Ahmad MF, Avruch J, Woodgett JR. (1994). The Stress-activated protein kinase subfamily of c-Jun kinases.*Nature.*12;369:56-60

Koch AE, Kunkel S, Harlow B, Johnson H, Evanoff G, Haines MD, Burdick RM, Pope and Streiter RM. (1992) Enhanced production of monocyte chemotactic protein -1 in rheumatoid arthritis. *J. Clin. Inv.* 90:772-779.

Kodaki T. Woscholski R. Hallberg B, Rodriguez-Viciana, Downward J, Parker P. (1994) The activation of phosphatidylinositol 3-kinase by Ras. *Curr.Biol.* 4, 798-806.

Kong M, Mournier C, Wu J, Posner B. (2000) Epidermal Growth Factor-induced Phosphatidylinositol 3-Kinase Activation and DNA Synthesis. *J.Biol.Chem.* 275:36035-42.

Kostensis E, Zeng F, Wess J. (1999). Structure-function analysis of muscarinic receptors and their associated G proteins. *Life Sci.* 64:355-362.

Kuang, Y. Wu, Y. Jiang, H. (1996). Selective G protein coupling by C-C chemokine receptors. *J Biol. Chem. 271, 3975-3978.*

Kumar V, Cotran R, Robbins S. (1997). Basic Pathology. 6th Ed. W.B Saunders.

Laemmli UK. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature.* 227:680-685.

Laudanna C, Mochly-Rosen D, Liron T, Constantin G, Butcher EC, (1998). Butcher that ξ protein kinase C involvement in polymorphonuclear neutrophil integrin-dependent adhesion and chemotaxis. *J. Biol. Chem.* 273: 30306-30315.

Lauffenburger DA, Horwitz AF (1996). Cell Migration: A Physically integrated molecular process. *Cell.* 84:359-369.

Lawlor M, Alessi D. (2001). PKB/Akt: a key mediator of cell proliferation, survival and insulin responses? *J. Cell. Sci.* 114:2903-2910.

Lee, C-W. Lee, H. Park, D. (1994) . *Journal of Biological Chemistry.* 268, 25335-8.

Lee B. (1998). Influence of the CCR2-V64I polymorphism on HIV-1 coreceptor activity and chemokine receptor function of CCR2b, CCR3, CCR5 and CXCR4. *J. Virol.* 72:7450-7458.

Legler DF, Loetscher M, Roos RS, Clark Lewis I, Baggiolini M, Moser B:(1998). B cell-attracting chemokine 1, a human CXC chemokine expressed in lymphoid tissues, selectively attracts B lymphocytes via BLR1/CXCR5. *J Exp Med* 187: 655-660,1998.

Le Good, J. A., Ziegler, W. H., Parekh, D. B., Alessi, D. R., Cohen, P., & Parker, P. J.: Protein kinase C isotypes controlled by phosphoinositide 3-kinase through the protein kinase PDK1. *Science* 281, 2042-5 (1998)

Lemmon M, Ferguson K. Signal-dependent membrane targeting by pleckstrin homology domains. *J. Biochem.* 350: 1-18.

Leopoldt D, Hanck T, Exner T, Maier U, Wetzker R, Nurnber B. (1998). Gβγ stimulates phosphoinositide 3-kinase-γ by direct interaction with two domains of the catalytic p110 subunit. *J.Biol.Chem.* 273:7024-7029

Levitan D, Greenwald I: (1995) Facilitation of lin-12-mediated signalling be sel-12, a Caenorhabditis elegans S182 Alzheimer's disease gene. *Nature* 377:351,

Li Z, Ziang H, Xie W, Zhang Z, Smrcka, Wu D. (2000) Roles of PLC-β_2 and β_3 and PI3Kγ in chemoattractant-mediated signal transduction. *Science*. 287: 1046-1049

Liang J, Kornfeld S. (1997). Comparative Activity of ADP-ribosylation Factor Family Members in the Early Steps of Coated Vesicle Formation on Rat Liver Golgi Membranes *J. Biol. Chem.* 272: 4141-4148.

Lilly C, Daugherty B. (2001). A novel LPS-Inducible CCR3 Activator. Why so many CCR3 ligands. *Am.J. Respir.Cell.Mol.Biol.* 25:673-675.

Lin P, Le-Niculescu H, Hofmeister R, McCaffery JM, Jin M, Hennemann H, McQuistan T, De Vries L, Farquahar M. (1998). The Mammalian Calcium-binding Protein, Nucleobindin (CALNUC), is a Golgi Resident Protein. *J. Cell. Biol.* 171(7): 1515-1527.

Lisovitch, M. Chalifa, V. (1999) *Signal-Activated Phospholipases* (Ed. Liscovitch, M.), R.G Landes, Austin, TX, pp 31-63.

Liscovitch, M; Czarny, M; Fiucci, G; Lavie, Y; Tang, X. (1999). Localization and possible functions of phospholipase D isozymes. *Biochim.Biophys.Acta*. 1439, 245-263.

Liu L, Damen j, Ware M, Huges M, Krystal G. (1997). SHIP, a new player in cytokine-induced signalling. *Leukemia*. 11 :181-184

Littman D. (1998). Chemokine Receptors. Keys to AIDS Pathogenesis? *Cell.* 93-677-680.

Logan SK, Falasca P, Hu P, Schlessinger J. (1997). Phosphatidylinositol 3-kinase mediates epidermal growth factor-induced activation of the c-Jun N-terminal kinase signalling pathway. *Mol. Cell. Biol.* 17: 5784-5790.

Lopez-Ilasaca M, Crespo P, Pellici P, Gutkind JS, Wetzker R, Wymann MP. (1998) Linkage of G-protein-coupled receptors to the MAPK signalling pathway through PI3-kinase-γ *Science.* 275:394-397.

Lu B, Rutledge BJ, Fiorello JA, Gu L, Gladie RP, Flavell RA, Rollins BJ. (1997). Transgenic MCP-1 in pancreatic islet produces monocyte-rich insulitis without diabetes. *J.Immunol.* 159:401-8.

Lu ZH. Berson JF, Chen YH, Turner J, Zhang TY, Sharron M, Harley Jenks M, Wang Z-X, Kim J, Rucker J, Hoxie J, Peiper S, Doms R. (1997). Evolution of HIV-1 coreceptor usage through interactions with distinct CCR5 and CXCR4 domains. *PNAS* . 94:4005-4010.

Lu, Z., Horna, A., Joseph, T., Sukezane, T., Frankel, P., Zhong, M., Bychenok, S., Xu, L., Feig, L. A., and Foster, D. A. (2000) Phospholipase D and RalA cooperate with the epidermal growth factor receptor to transform 3Y1 rat fibroblasts.*Mol. Cell. Biol.* 20, 462-467

Lubkowski J, Bujacz G, Boque L, Domaille P, Handel T, Wlodeawer A. (1997). The molecular structure of MCP-1 in 2 crystal form provides a rare example of variable quaternary interactions. *Nat. Struct. Biol.* 4:64-69.

Lukacs NW. Kunkel SL. (1998). Chemokines and their role in disease. *J Clin Lab. Res.* 28 :91.

Lu-Kuo J, Fruman D, Joyal D, Cantley L, Katz H. (2000). Impaired kit- but not FcεRI-Initiated mast cell activation in the absence phosphoinositide 3-kinase p85α gene products. *J. Biol. Chem.* 275, 6022-6029.

Luster A. (1998). Chemokine - Chemotactic Cytokines that mediate Inflammation. *New. Eng. J. Med.* 338:436-445.

Luttrell LM, Hawes BE, van Biesen T, Luttrell DK, Lansing TJ, Lefkowitz RJ. (1996) Role of c_Src tyrosine kinase in G protein-coupled receptor- and Gβγ subunit-mediated activation of mitogen activated protein kinases. *J. Biol. Chem.* 271:19443-19450.

Ma YC, Huang J, Ali S, Lowry W Huang XY. (2000) Src tyrosine kinase is a direct novel effector of G proteins. *Cell.* 102:635-646.
Ma Q, Jones D, Borghesani PR, Segal RA, Nagasawa T, Kishimoto T, Bronson RT, Springer TA: (1998). Impaired B-lymphopoiesis, myelopoiesis, and derailed cerebellar neuron migration in CXCR4- and SDF-1-deficient mice. *Proc Natl Acad Sci USA* 95:9448 –9453.

Maehamer T and Dixon JE. (1998). The tumour suppressor, PTEN/MMAC1, dephosphorylates the lipid second messenger, phosphatidylinositol 3,4,5-trisphosphate. *J. Biol. Chem.* 273:13375-8.

MacKay D, Hall A. (1998). Rho GTPases. *J. Biol. Chem.* 273:20685-20688.

Madrid LV, Mayo MW, Reuther JY, Baldwin AS. (2001). Akt stimulates the transactivation potential of the RelA/p65 Subunit of NF-kappa B through utilization of the Ikappa B kinase and activation of the mitogen-activated protein kinase p38. *J Biol Chem.* 276(22):18934-40.

Maira S, Glaetic I, Brazil D, Kaech S, Ingley E, Thelen M, Hemmings B. (2001). Carboxy-terminal modulator protein (CTMP), a negative regulator of PKB/Akt and v-Akt at the plasma membrane. *Science.* 294. Oct.

Mantovani A. (1994) Biology of disease, tumour associated macrophages in neoplastic progression: a paradigm for the *in vivo* function of chemokines. *Lab. Invest.* 71:5-16.

Mantovani A. (1999). The chemokine system: redundancy for robust outputs. *Imm.Today.* 20: 254-257

Marie C, Roman-Roman S, Rawadi G. (2001) Involvement of Mitogen-Activated Protein Kinase Pathways in Interleukin-8 Production by Human Monocytes and polymorphonuclear Cells Stimulated with Lipopolysaccharide or *Mycoplasma fermentans*. *Infect. Immun.* 67: 688-693.

Marra F, Romanelli R, Giannini C, Failli P, Pastacaldi S, Arrighi MC, Pinzani M, Laffi G, Montalto P, Gentilini P. (1999) Monocyte Chemotactic Protein-1 as a Chemoattractant for Human Hepatic stellate Cells. *Hepatology*. 29:140-148.

Marshall CJ. (1995). Specificity of receptortyrosine kinase signalling:transient versus sustained extracellular signal-related kinase activation. *Cell*. 80:179-185.

Matsuoka I, Mori T, Aoki J, Sato T, Kurihara K (1993). Identification of novel members of G-protein coupled receptor superfamily expressed in bovine taste tissue *Biochem. Biophys. Res. Commun.* 194: 504-511.

Mebius, R. E., P. R. Streeter, S. Michie, E. C. Butcher, I. L. Weissman. (1996). A developmental switch in lymphocyte homing receptor and endothelial vascular addressin expression regulates lymphocyte homing and permits $CD4^+$ $CD3^-$ cells to colonize lymph nodes. *PNAS.* 93:11019

McPhail, LC. Waite, K. Reier, D. (1999). A novel protein kinase target for the lipid second messenger phosphatidic acid. *Biochim.Biophys.Acta*. 1439, 277-290

Metjian, A. Roll, R. Ma, A. Abrams, C. (1999). Agonists Cause Nuclear translocation of Phosphatidylinositol 3-Kinase-γ *J.Biol.Chem*. 274, (39) 27943-27947.
exchange factor, and rac. *Mol.Cell.Biology*. 18:4744-4751

Meili R, Ellsworth C, Lee S, Reddy TBK, Ma H, Firtel R. (1999). Chemoattractant-mediated transient activation and membrane localisation of PKB is required for efficient chemotaxis to cAMP in *Dictyostelium*. *EMBO*. 18 :2092-2105.

Mellado, M. Rodriguez-Frade, J. Aragay, A. del Real, G, Martin A, Vila-Coro A, Serrano A, Mayor F, Martinez-A. (1998) Roles of Chemokines and Receptor Polarization in NK-Target Cell Interactions. *J.Immunol*. 161:805-813.

Mellado M, Rodriguez-Frade M, Manes S, Martinez-A C. (2001). Chemokine Receptor signalling and functional responses: the role of dimerisation and tyrosine kinase pathway activation. *Ann. Rev. Immunol.* 19:397-421.

Mellado M, Rodriguez-Frade M, Coro-Vila A, Fernandez S, Martin de Ana A, Jones D, Toran, Martinez-A C. (2001). Chemokine Receptor homo- or heterodimerisation activates distinct signalling pathways. *EMBO*. 20:2497-2507.

Mellado, M. Rodriguez-Frade, J. Aragay, A. del Real, G, Martin A, Vila-Coro A, Serrano A, Mayor F, Martinez-A. (1999). Chemokine control of HIV infection. *Nature* 400, 723 – 724.

Mercurio, A and Shaw, L. (1988). Macrophage interactions with laminin: PMA selectively induces the adherence and spreading of mouse macrophages on a laminin substratum. *J Cell Biol.* 107:1873-1880.

Middleton, J., S. Neil, J. Wintle, I. Clark-Lewis, H. Moore, C. Lam, M. Auer, E. Hub, A. Rot. (1997). Transcytosis and surface presentation of IL-8 by venular endothelial cells. *Cell 91:385.*

Miller JR, Moon RT. (1996). Signal transduction through beta-catenin and specification of cell fate during embryogenesis. *Genes and Development.* 10:2527-2539.

Mirmonsef P, Shelburne C, Fitzhugh C, Yeatman, II, Chong HJ, and Ryan J. (1999). Inhibition of Kit Expression by IL-4 and IL-10 in Murine Mast Cells: Role of STAT6 and Phosphatidylinositol 3'-Kinase *J Immunol* 1999 163: 2530-2539.

Mochly-Rosen D, Henrich CJ, Cheever L, Khaner H, Simpson PC. (1990). A protein kinase C isoenzyme is translocated to cytoskeletal elements on activation. *Cell Regul.* 1: 693-706.

Monteclaro F, Arai H, Charo I. (1997). Molecular Approaches to Identifying Ligand Binding and Signalling Domains of CC Chemokine Receptors. *Methods.Enzymol.* 288:70-86.

Morgan SJ, Smith AD, Parker PJ (1990). Purification and characterisation of bovine brain type I phosphatidylinositol kinase. *Eur. J. Biochem.* 191, 761-767.

Morris A, Malbon C. (1999). Physiological Regulation of G Protein-Linked Signaling. *Physiol Rev.* 79::1373 - 1430

Moyer CF, Sajuthi D, Tulli H, Williams JK. Synthesis of IL-1α and IL-1β by arterial cells in atherosclerosis. *Am. J. Pathol.* 138:951-960

Munchi N, Ganju RK, Avraham S, Mesri E, Groopman J. (1999). Kaposi's Sarcoma-associated Herpesvirus-encoded G Protein-coupled Receptor Activation of c-Jun Amino-terminal Kinase/Stress-activated Protein Kinase and Lyn Kinase Is Mediated by Related Adhesion Focal Tyrosine Kinase/Proline-rich Tyrosine Kinase 2. *J. Biol. Chem.* 274: 31863-31867.

Murdoch C and Finn A. (2001). Chemokine Receptors and their role in inflammation and infectious diseases. *Blood.* 95(10):3032-3043.

Murphy PM, Baggiolini M, Charo IF, Herbert CA, Horuk R, Matsushima K, Miller LH, Openheim JJ, Power CA. (2000). International Union of Pharmacology. XXII. Nomenclature for Chemokine receptors. *Pharmacol. Rev.* 52:145-176.

Myers SJ, Wong LM, Charo IF. (1995). Signal transduction and ligand specificity of the human monocyte chemoattractant protein-1 receptor in transfected embryonic kidney cells.*J. Biol. Chem.* 270:5786-5792.

Nelken, N. Coughlin, S. Gordon D, Wilkox JN. (1991). Monocyte-chemoattractant protein-1 in human atheromatous plaques. *J. Clin. Invest.* 88, 1121-1127.

New L, Jiang Y, Zhao M, Liu K, Flood LJ, Kato Y, Parry GCN, Han H. (1998) PRAK, a novel protein kinase regulated by the p38 MAP kinase.*EMBO J.* 17:3372-3384.

Neer EJ. (1995) Heterotrimeric G proteins :organisers of transmembrane signals. Cell. 80:249-57.

Neptune E, Bourne. (1997) Receptors induce chemotaxis by releasing the $\beta\gamma$ subunit of G_i, not by activating G_q or G_s. *Proc. Natl. Acad. Sci.* 94:14489-14494.

Neptune E, Liri T, Bourne H. (1999) $G\alpha_i$ is not required for chemotaxis mediated by $G\alpha_i$-coupled receptors. *J. Biol. Chem.* 274:2824-2828.

Nibbs R, Wylie S, Pragnell I Graham G. (1997a). Cloning and characterisation of novel murine β chemokine receptor, D6. *J. Biol. Chem.* 272: 12495-12504.

Nibbs, R. Wylie, SM. Yang J. Graham, G. (1997b). Cloning and Characteriation of a Novel Murine C Chemokine Receptor, D6. *J.Biol.Chem.* 272 (51) 32078-83.

Nibbs, R.J.B., Kriehuber, E., Ponath, P.D., Parent, D., Qin, S., Campbell, J.D.M., Henderson, A., Kerjaschki, D., Maurer, D., Graham, G.J. and Rot, A. (2001) The b-chemokine receptor D6 is expressed by lymphatic endothelium and a subset of vascular tumours. *Am. J. Pathol.* 158, 867-877

Nieto M, Frade J, Sancho D, Mellado M, Martinez-A, Sanchez-Madrid. (1997). Polarisation of chemokine receptors to the leading edge during lymphocyte chemotaxis. *J. Exp. Med.* 186:153-158.

Odorizzi G, Babst M, Emr SD (2000). Phosphoinositide signaling and the regulation of membrane trafficking in yeast. *Trends in Biochemical Sciences.* 25:299-35.

Ohguchi, K (1999). Phospholipase D development during differentiation of human promyelocytic leukemic HL60 cells. *Biochim.Biophys.Acta.* 1439, 215-227

Okada T, Sakuma Y, Fukui O, Hazeki M, Ui. (1994). Blockage of chemotactic peptide-induced stimulation of neutrophils by wortmannin as a result of selective inhibition of phosphatidylinositol 3-kinase. *J.Biol.Chem.* 269:3563.

Oppenheim J, Zacharie C, Mukaida N, Matsushima K (1991). Properties of the novel proinflammatory supergene 'intercrine' cytokine family. *Annu.Rev.Immunol.* 9:617-648.

Palsson E, Popoff M, Thelestam M, O'Neil L. (2000). Divergent roles for Ras and Rap in the activation of p38 Mitogen-activated Protein Kinase by Interleukin-1. *J. Biol. Chem.* 275:7818-7825.

Pap M, Cooper G. (1998). Role of glycogen synthase kinase-3 in the phosphatidylinositol 3-kinase/Akt cell survival pathway. *J.Biol.Chem.* 273:19929-19932.

Parekh D, Ziegler W, Parker P. (2000). Multiple Pathways control protein kinase C phosphorylation. *EMBO J.* 19(4):496-503.

Parker P. (1995). PI 3-kinase puts GTP on the Rac. *Curr. Biol.* 5(6):577.

Pati S, Cavrois M, Guo H, Foulke J, Kim J, Feldman R, Reitz M. (2001). Activation of NF-κB by the Human Herpesvirus8 Chemokine Receptor ORF74: Evidence for the Paracrine Model of Kaposi's Sarcoma Pathogenesis. *J. Virol.* 75:8660-8673.

Patki V, Lawe D, Corvera S, Virbasius J, Chawla A. (1998). A Functional PtdIns(3)P-binding motif. *Nature* 394:433-434

Penton-Rol, G., Cota, M., Polentarutti, N., Luini, W., Bernasconi, S., Borsatti, A., Sica, A., LaRosa, G.J., Sozzani, S., Poli, G., Mantovani, A: (1999). Up-regulation of

CCR2 chemokine receptor expression and increased susceptibility to the multitropic HIV strain 89.6 in monocytes exposed to glucocorticoid hormones. *J. Immunol.*, 163:3524-3529.

Peters W and Charo I (2001). Involvement of chemokine receptor-2 and its ligand, monocyte-chemotactic protein-1, in the development of atherosclerosis. *Curr. Opin. Lipid.* 12:175-180.

Petit & Hoffbrand. (1992) Essential Haematology. 3rd Edition. (Blackwell Science).

Ping D, Jones PL, Boss JM. (1996). TNF regulates the in vivo occupancy of both distal and proximal regulatory regions of the MCP-1/JE gene. *Immunity.* 4:455-469.

Polentarutti N, Introna M, Sozzani S, Mancinelli R, Mantovani G, Mantovani A. 1997. Expression of monocyte chemotactic protein-3 in human monocytes and endothelial cells. *Eur. Cytokine Netw.* 8: 271.

Ponting C, Parker P (1996). Extending the C2 domain family. *Prot. Sci.* 5:162-166.

Popoff M, Chaves-Olarte E, Lemichez E, von Eichel-Streiber C, Thelestam M, Chardin P, Cussac D, Antonny B, Chavrier P, Flatau G, Giry M, de Gunzburg J, Boquet P. (1996). Ras, Rap and Rac small GTP-binding proteins are targets for *Clostridium* sordelli lethal toxin glucosylation. *J. Biol. Chem.* 271:10217-10224.

Prasad KV, Janssen O, Kapeller R, Raab M, Cantley L, Rudd CE. (1993). PNAS Src-Homology 3 Domain of Protein Kinase $p59^{fyn}$ Mediates Binding to Phosphatidylinositol 3-Kinase in T Cells. *PNAS.* 90:7366-70.

Rameh LE, Chen CS, Cantley LC. (1995) Phosphatidylinositol(3,4,5)P_3 interacts with SH2 domains and modulates PI-3-kinase association with tyrosine-phosphorylated proteins Cell; 83: 821-830

Ransohoff R, Hamilton TM, Tani M, Stoler MH, Schick HE, Major JA, Estes ML, Thomas DM, Tuohy VK. (1993). Astrocyte expression of mRNA encoding cytokines IP-10 and JE/MCP-1 in experimental autoimmune encephalomyelitis. *FASEB.* 7:592-600.

Rebollo A, Martinez-A C. (1999). Ras Proteins: Recent advances and new functions. *Blood.* 94(9):2971-2980.

Reif K, Cantrell D. (1998). Networking Rho family GTPases in Lymphocytes. *Immunity.* 8:395-401.

Rahman A, Anwar KN, Uddin S, Xu N, Ye RD, Platanias LC, Malik AB. (2001). Protein kinase C-delta regulates thrombin-induced ICAM-1 gene expression in endothelial cells via activation of p38 mitogen-activated protein kinase. *Mol Cell Biol.* 2001 21(16):5554-65.

Rhee, S and Bae, Y. (1997). Regulation of phosphoinositide-specific phospholipase-C isoforms. *J.Biol.Chem* 272:15045-15048.

Rizzo, M. Shome, K *et al.* (1999). *Journal of Biological Chemistry.* 274 (2) 1131-1139.

Rogaev EI, Sherrington R, Rogaeva EA, Levesque G, Ikeda M, Liang Y, Chi H, Lin C, Holman K, Tsuda T, Mar L, Sorbi S, Nacmias B, Placentini S, Amaducci L, Chumakov I, Cohen D, Lannfelt L (1995). Familial Alheimer's disease in kindreds with missense mutations in a gene on chromosome 1 related to the Alzhelmer's disease type 3 gene. *Nature* 376:775,

Rohrschneider I, Fuller J, Wolf I, Liu Y, Lucas D. (2000). Structure, function and biology of SHIP proteins. *Genes.Dev.* 14:505-520.

Rollins B, Morton C, Ledbetter DH, Eddy RL, Shows TB. (1991). Assignment of the human small inducible cytokine A2 gene, SCYA2 (encoding JE or MCP-1), to 19q11.2-12: evolutionary relatedness of cytokines clustered at the same locus. *Genomics*. 10 (2): 489-492.

Rommel,C. Hafen, E. (1998). Ras – versatile cellular switch. *Curr.Opin.Gen.Dev.* 8, 412-418.

Rothenberg ME, MacLean JA, Pearlman E, Luster AD, Leder P (1997): Targeted disruption of the chemokine eotaxin partially reduces antigen-induced tissue eosinophilia. *J Exp Med* 185:785 -790, 1997

Saito K, Scharenberg A, Kinet JP. (2001). Interaction between the Btk PH Domain and Phosphatidylinositol-3,4,5-trisphosphate Directly Regulates Btk *J. Biol. Chem.* 276: 16201-16206.

Salcedo R, Ponce M, Young H, Wasserman K, Ward J, Kleinman H, Oppenheim J, Murphy W. (2000). Human endothelial cells express CCR2 and respond to MCP-1: direct role of MCP-1 in angiogenesis and tumor progression. *Blood.* 96(1):34-40.

Sasaki T, Sasaki J, Jones R, Oliveira-dos-Santos A, Stanford W, Bolon B, Wakeham A, Itie A, Bouchard D, Kozieradzki I, Joza N, Mak T, Ohashi P, Suzuki A, Penninger J. (2000). Function of PI3Kγ in thymocyte development, T Cell activation and neutrophil migration. *Science*. 287:1040-1045.

Samson M, La Rosa G, Libert F, Paindavoine P, Detheux M, Vassart G, Parmentier. (1997). The second extracellular loop of CCR5 is the major determinant of ligand specificity. *J. Biol. Chem.* 272:24934-24941.

Sanchez-Madrid F, Angel del Pozo. (1999) Leukocyte polarisation in cell migration and immune interaction. *EMBO*. 18:501-511

Sarau H, Rush J, Foley J, Brawner M, Schmidt D, White J, Barnette M. (1997). Characterisation of Functional Chemokine Receptors (CCR1 and CCR2) on Eol-3 Cells: A Model System to Examine the Role of Chemokines in Cell Function. *J. Pharm. Exp. Therap.* 283: 411-418.

Schall TJ and Bacon K. (1994) Chemokines, Leukocyte trafficking and inflammation. *Curr. Opinion Immunol.*. 6:865-873.

Schofield L, Novakovic S, Gerold P, Schwarz RT, McConville MJ, Tachado SD. (1996). Glycophosphatidylinositol toxin of *Plasmodium* up-regulates intercellular adhesion molecule-1, and E-selectin expression in vascular endothelial cells and

increases leukocyte and parasite cytoadherence via tyrosine kinase-dependent signal transduction. *J.Immunol.* 156:1886-1896.

Schroder JM. Noso N, Sticherling M, Christophers E. (1996). Role of eosinophil-chemotactic-C-C chemokines in cutaneous inflammation. *J Leukoc. Biol.* 59 :1.

Schweickart V, Epp A, Raport C, Gray P. (2000). CCR11 is a functional receptor for the monocyte Chemoattractant Protein Family of Chemokines. *J.Biol.Chem.* 275 :9550-9556.

Sebastiani S. Allavena P, Albanesi C, Nasorri F, Bianchi G, Traidl C, Sozzani S, Girolomoni G, Cavani A. (2001). Chemokine receptor Expression and Function in CD4+ T lymphocytes with Regulatory Activity. *J Immunol.* 166 :996-1002.

Serger, R. Krebs,E. (1995). *FASEB.* 9, 726-735.

Schall TJ and Bacon K. (1994). Chemokines, leukocyte trafficking, and inflammation *Curr. Opin. Immunol.* 6:865

Shecter AD, Rollins BJ, Zhang YJ, Charo IF, Fallon JT, Rossikhina M, Giesen PL. (1997). Tissue Factor is Induced by Monocyte Chemoattractant Protein-1 in Human Aortic Smooth Muscle and THP-1 Cells. *J. Biol. Chem.* 272:28568-28573.

Shepard P, Nave O' Rahilly. (1996). The role of PI3-kinase in Insulin signalling. *J.Mol. Endo.* 17, 17869-

Sherrington R, Rogaev EI, Liang Y, Rogaeva EA, Levesque G, Ikeda M, Chi H, Lin C, Li G, Holman K, Tsuda T, Mar L, Foncin J-F, Bruni AC, Montesi M.P., Sorbi S, *et* al. (1995) Cloning of a gene bearing missense mutations in early onset familial Alzheimer's disease. *Nature* 375:754

Shyamala V, Khoja H. (1998). Interleukin-8 receptors R1 and R2 activate mitogen-activated protein kinases and induce c-fos independent of Ras and raf-1 on Chinese Hamster Ovary cells. *Biochemistry.* 37:15918-15924.

Shyy Y, Hsieh J, Usami S, Chien S. (1994). Fluid Sheer Stress Induces Biphasic Response of Human Monocyte Chemotactic Protein-1 Gene Expression in Vascular Endothelium. *PNAS .* 91:4678

Sica A, Saccani A, Bottazzi B, Bernasconi S, Allavena P, Gaetano B, Fei F, LaRosa G, Scotton C, Balkwill F, Mantovani A. (2000). Defective expression of the Monocyte chemotactic protein-1 receptor CCR2 in macrophages associated with human ovarian carcinoma. *J.Immunol* 164:733-738.

Siddiqui RA, English D. (1997). Phosphatidic acid elicits calcium mobilization and actin polymerization through a tyrosine kinase-dependent process in human neutrophils: a mechanism for induction of chemotaxis. *Biochim Biophys Acta.* 8;1349(1):81-95.

Siegfried E, Chou T, Perrimon N (1992). Wingless signalling acts through *zeste-white 3*, the Drosophila homologue of glycogen synthase kinase-3, to regulate engrailed and establish cell fate. *Cell.* 71:1167-1179.

Signoret N Oldridge, Pelchen-Matthews, Klasse P, Tran T, Brass L, Rosenkilde M, Schwartz T, Holmes W, Dallas W, Luther M, Wells T, Hoxie J, Marsh M. (1997).

Phorbol esters and SDF-1 induce rapid endocytosis and down modulation of chemokine receptor CXCR4. *J. Cell. Biol.* 139:651-664.

Siveke, J., and A. Hamann.(1998). Cutting edge: Th1 and Th2 cells respond differentially to chemokines. *J.Immunol.*:160:550-554.

Simpson *et al.* (2000). Expression of β-chemokine receptors CCR2, CCR3, and CCR5 in multiple sclerosis central nervous system tissue. *J.Neuroimmunnol.* 108:192-200.

Slaaby, R., Jensen, T., Harald, H. S., Frohman, M. A., and Seedorf, K. (1998) PLD2 complexes with the EGF receptor and undergoes tyrosine phosphorylation at a single site upon agonist stimulation. *J. Biol. Chem.* 273, 33722-33727.
Sonnenburg E, Gao T, Newton A. (2001). The Phosphoinositide-dependent Kinase, PDK-1,Phosphorylates Conventional Protein Kinase C Isozymes by a Mechanism That Is Independent of Phosphoinositide 3-Kinase. *J. Biol. Chem.*, Vol. 276, Issue 48, 45289-45297.

Sorenson T *et al*, (1999) Expressions of specific chemokines and chemokine receptors in the central nervous system of multiple sclerosis patients. *J. Clin. Invest.* 103:807-815.

Sotsios Y, Ward SG. (2000). Phosphoinositide 3-kinase: a key biochemical signal for cell migration in response to chemokines. *Immun. Rev.*

Sotsios Y, Whittaker GC, Westwick J, Ward SG. (1999). The CXC Chemokine stromal cell-derived factor activates a Gi-coupled phosphoinositide 3-kinase in T lymphocytes. *J. Immunol.* 163: 5954.

Sozzani S, Molino M, Locati M, Luini W, Cerletti C, Vecchi A, Mantovani A. (1993) Receptor-activated calcium influx in human monocytes exposed to monocyte chemotactic protein-1 and related cytokines.. *J. Immunol.* 150:1544-1553.

Sozzani S, Ghezzi S, Iannolo G, Luini W, Borsatti M, Polentarruti N, Sica M, Locatti T, Mackay C, Wells T. (1998). Interleukin-10 increases CCR5 expression and HIV infection in monocytes. *J. Exp.Med.* 187:439.

Sozzani S, Zhou, Locati M, Rieppi M, Proost M, Magasin M, Vita N, van Damme J, Mantovani M. (1994). Receptors and transduction pathways for monocyte chemotactic protein-2 and monocyte chemotactic protein-3. Similarities and differences with MCP-1. *J.Immunol.* 7:3615-3622.

Springer TA. (1994) Traffic Signals for Lymphocyte Recirculation and Leukocyte Emigration - The Multistep Paradigm. *Cell.* 76(2):301-314.

Stellato, C. Collins, P. Ponath P, Soer D, Newman W, La Rosa G, LI H, White J, Schwiebert LM, Bickel C, Liu M, Bochner BS, Williams T, Schleimer P (1997). Production of the Novel C-C chemokine MCP-4 by airway cells and comparison of its biologicAL activity to other C-C chemokines. *J. Clin.Invest..* 99 (5), 926-36.

Stephens, L. Hughes, KT, Irvine RF. (1991). Pathway of Phosphatidylinositol(3,4,5)-trisphosphate synthesis in activated Neutrophils. *Nature.* 351, 33-39.

Stephens L, Eguinoa A, Corey S, Jackson T, Hawkins PT. (1993). Receptor stimulated accumulation of phopshatidylinositol (3,4,5)-trisphosphate by G-protein mediated pathways in human myeloid derived cells. *EMBO J.* 12:2265

Stephens, L. Jackson, T. Hawkins, P. (1993). Agonist-stimulated synthesis of Phosphatidylinositol (3,4,5)-trisphosphate - a new intracellular signalling system. *Biochim. Biophys. Acta.* 1179, 27-75.

Stephens L, Smrcka A, Cooke FT, Jackson TR, Sternweis PC, Hawkins PT. (1994). A Novel polyphosphoinositide 3 kinase activity in Myeloid-derived cells is activated by G protein βγ subunits. *Cell.* 77:83-93.

Stephens, L.R., Eguinoa, A., Erdjument-Bromage, H., Lui, M., Cooke, F., Coadwell, J., Smrcka, A.S., Thelen, M., Cadwallader, K., Tempst, P., and Hawkins, P.T. (1997) The Gbγ sensitivity of a PI3K is dependent upon a tightly associated adaptor, p101. *Cell* 89, 105-114

Stoker H, Andjelkovic M, Oldham S, Laffargue M, Wymann M, Hemmings B, Hafen E. (2002). Living with lethal PIP3 levels: Viability of flies lacking PTEN restored by a PH domain mutation in Akt/PKB. *Science.* 295: March.

Stoyanov B, Volinia S, Hanck T, Rubio I, Loubchenkov M, Malek D, Stoyanova S, Vanhaesebroeck B, Dhnad R, Nurnberg P, Gierschik P, Seedorf K, Hsuan J, Waterfield M, Wetzker R. (1995) Cloning and Characterisation of a G-protein activated human phoshatidylinositide 3-kinase. *Science.* 269:690.

Sullivan S, McGrath D, Liao F, Boehme S, Farber J, Bacon K. (1999). MIP-3α induces human eosinophil migration and activation of the mitogen-activated protein kinases (p42/p44 MAPK). *J. Leuk. Biol.* 66:674-682.

Suzuki H, Freije D, Nusskern D, Okami K, Cairns P, Sidransky D, Issacs WB, Bova GS. (1998). Interfocal heterogeneity of PTEN/MMAC1 gene alterations in multiple metastatic prostate cancer tissues. *Cancer Res.* 58:204-209.

Swillens S, Waelbroek M, and Champeil P. (1995). Does radiolabelled ligand bind to a homogenous population of non-interacting receptor sites? TIPS 16:151-155.

Szalai C, Duba J, Prohaszka Z, Kalina A, Szabo T, Nagy B, Horvath L, Csaszar A. (2001). Involvement of polymorphisms in the chemokine system in the susceptibility for coronary artery disease (CAD). Coincidence of elevated Lp(a) and MCP-1 – 2518 G/G genotype in CAD patients. *Atherosclerosis.* 158:233-239.

Tachibana K, Hirota S, Iizasa H, Yoshida H, Kawabata K, Kataoka Y, Kitamura Y, Matsushima K, Yoshida N, Nishikawa S, Kishimoto T, Nagasawa T: (1998). The chemokine receptor CXCR4 is essential for vascularization of the gastrointestinal tract. *Nature.* 393 : 591-594.

Taha RA, Laberge S, Hamid Q, Olivenstein R. (2001). Incresed Expression of Chemoattractant Cytokines Eotaxin, MCP-2 and IL-16 in Induced Sputum from Asthmatic Patients. *Chest.* 120:595-601.

Tamura M, Gu J, Yamada K. (1999). PTEN gene and integrin signalling in cancer. *J. Nat. Canc. Inst.* 91:1820-1828.

Takeda, H. Matozaki, T Takada T, Noguchi T, Yamao T, Tsuda M, Ochi F, Fukunaga K, Inagaki K, Kasuga M. (2000). PI 3-kinase γ and protein kinase C-ζ mediate RAS-independent activation of MAP kinase by a G_i protein-coupled receptor *EMBO J.* 18 (2), 386-395.

Tedder TF. (1995). The Selectins: Vascular adhesion molecules. *FASEB J.* 9:866

Terry RW, Kwee L, Levine JF, Labow MA. (1993). Cytokine induction of an alternatively spliced murine vascular cell adhesion molecule (VCAM) mRNA encoding a glycosylphosphatidylinositol-anchored VCAM protein. *Proc. Natl Acad. Sci USA.* 90:5919-5923.

Thelen M. (2001). Dancing to the tune of chemokines. *Nature. Imm.* 2:129-134.

Tilton B, Andejelkovic M, Didichenko S, Hemmings B, Thelen M (1997). G protein-couple receptors and Fcγ-rceptors mediate activation of Act protein kinase B in human phagocytes. *J.Biol.Chem.* 272:28096-28101.

Tilton B *et al.* (2000). Signal Transduction by CXC chemokine receptor 4. Stromal cell derived factor-1 stimulates prolongued protein kinase B and extracellular signal-regulated kinase 2 in T lymphocytes. *J. Exp. Med.* 192:313-324.

Tolias KF, Cantley L, Carpenter CL. (1995) Rho family GTPases bind to Phosphoinositide kinases. *Journal of Biological Chemistry.* 270:17656-17659.

Tsai M, Hall A, Stacey DW. (1989). Inhibition by phospholipids of the interaction between R-ras, rho, and their GTPase-activating proteins. *Mol. Cell. Biol.* 9: 5260-5264

Tsuchiya, S. Yamabe, M. Yamaguchi, Y, Kobayashi, Y. Konno, T. Tada, K (1980) *International Journal of Cancer.* 26, 171-176

Tsuchiya, S. Goto, Y. Kobayashi, Y. Okumura, H Konno, T. Tada, K (1982) *Cancer Research. 42,* 1530-1536.

Turner L, Ward SG, Westwick J. (1995) RANTES-activated human T lymphocytes: a role for phosphatidylinositide 3-kinase. *J. Immunol.* 155:2437-2444

Turner, SJ. Domin, J. Waterfield, M. Ward, SG. Westwick, J. (1998). The CC Chemokine Monocyte Chemokine Peptide-1 Activates both the Class I p85/p110 Phosphatidylinositol 3-kinase and the Class II PI3K-C2α. *J.Biol.Chem..* 273 (40), 25987-25995.

Tsokos GC. (1995). Lymphocytes, cytokines, inflammation and immune trafficking. *Curr. Opin Rheumatolol. 7*:376.

Ueno Tm Toi M, Saji H, Muta M, Bando H, Kuroi K, Koike M, Inadera H, Matsushima K. (2000). Significance of Macrophage Chemoattractant Protein-1 in Macrophage Recruitment, Angiogenesis and Survival in Human Breast Cancer. *Clin. Can. Res.* 6:3282-3289.

Valente A, Xie F, Abramova M, wenzel U, Abboud H, Graves DT. (1998). A Complex Element Regulates IFN--Stimulated Monocyte Chemoattractant Protein-1 Gene Transcription. *J. Immunol.* 161:3719-3728.

Van Damme J, Proost P, Lenarts J, Opdenker G (1992) Structural and functional identification of two human, tumor-derived monocyte chemotactic proteins (MCP-2 and MCP-3) belonging to the chemokine family. *J. Exp. Med.* 176:59-65.

Vanhaesebroeck B, Leevers S, Panaytou G, Waterfield MD. (1997) Phosphoinositides 3-kinase:conserved family of signal transducers. *Trends in Biochemical Sciences.* 22:267-271.

Vanhaesebroeck B, Welham MJ, Kotani K, Stein R, Warne PH *et al.* (1997). p110δ, A Novel Phosphoinositide 3-kinase in Leukocytes. *Proceedings of the National Academy of Sciences USA.* 94:4330.

Vanhaesebroeck B, Waterfield M. (1999). Signalling by distinct classes of phosphoinositide-3-kinases. *Exp.Cell.Res.* 253:239-54.

Vanhaesebroeck B, Allessi D. (2000). The PI3K–PDK1 connection: more than just a road to PKB. *Biochem.J.* 561-576.

Vanhaesebroeck, B., Leevers, S.J., Ahmadi, K., Timms, J., Katso, R., Driscoll, P.C., Woscholski, R., Parker, P.J., and Waterfield, M.D. (2001) Synthesis and function of 3-phosphorylated inositol lipids. *Annu. Rev. Biochem.* 70, 535-602.

Venkateswarlu K, Oatey PB, Tavare JM, Cullen PJ. (1998) Insulin-Dependent translocation of ARNO to the plasma membrane of adiposites requires phosphatidylinositol 3-kinase. *Curr. Biol.* 8:463-466.

Virbasius JV, Guilherme A, Czech MP. Mouse p170 is a novel phosphoinositide 3-kinase containing a C2 domain. *JBiol.Chem..* 271: 13304-13307.

Voest, E., Kenyon,, O'Reilly MS, Truitt G, D'Amato RJ, Folkman J.(1995). Inhibition of angiogenesis in vivo by interleukin 12. *J. Natl. Cancer Inst.* 87:581.

Voice JK, Klemke R, Le A, Jackson JH. (1999). Four human homologs differ in their ability to activate Raf-1, induce transformtion, and stimulate cell motility. *J. Bil. Chem.* 274:17164-17170.

Vojtek A, Der C. (1998). Increasing Complexity of the Ras Signalling Pathway. *The J.Biol.Chem.* 19925-19928.

Wan Y, Kurosaki T, Huang X. (1996). Tyrosine kinases in activation of the MAP kinase cascade by G-protein-coupled receptors. *Nature.* 380:541-544.

Wang D, Richmond A. (2001). Nuclear factor-κB activation by the CXC chemokine melanoma growth-stimulatory activity/growth-regulated protein involves the MEKK1/p38 Mitogen-activated protein kinase pathway. *J. Biol.Chem.* 276:3650-3659.

Wang J, Park IW, Groopman JE. (2001). Stromal cell-derived factor-1 stimulates tyrosine phosphorylation of multiple focal adhesion proteins and induces migration of hematopoietic progenitor cells: roles of phosphoinositide-3kinase and protein kinase C. *Blood.* 95: 2505-2505.

Wang, K. Roufogalis, B. (1999). Ca2+/calmodulin stimulates GTP binding to the ras-related protein ral-A. *J.Biol.Chem.* 274, 14525-14528.

Ward SG, Westwick J, Hall ND, Sansom DM (1993) Ligation of CD28 receptor by B7 induces formation of D-3 phosphoinositides in T lymphocytes independently of T cell receptor/CD3 activation. Eur.J.Immunol. 10:2572-2577

Ward SG, June C, Olive D. (1996). PI 3-kinase: a pivotal pathway in T-cell activation? *Immunol.Today.* 17, 187-197.

Ward SG, Westwick J. (1998). Chemokines: Understanding their role in T lymphocyte biology. *Biochem. J.* 333:457-470.

Ward, SG. (1999). Measurement of Phopshoinositide 3-Kinase Activity. *Methods.Mol.Biol.* 1999. 138:163.

Ward S, Bacon K, Westwick J. (1998). Chemokines and T lymphocytes: more than an attraction. *Immunity.* 9:1-20.

Waterhouse C. Joseph R. Winsor G. Lacombe T Stadnyk A. W Kanke T. MacFarlane, S, Seatter MJ, Davenport E, Scott G, Hunter G, Paul, A, Plevin, R. Differential regulation by PKC isotypes in activation of c-Jun N-terminal kinase (JNK) and p38 MAP kinase mediated by proteinase-activated receptor-2 (PAR-2).

Weber KSC, Klickstein LB, Weber C. (1998). Chemokine-Induced Monocyte transmigration requires Cdc42-mediated cytoskeletal changes. *Eur.J. Immunol.* 28:2245-2251.

Weber, M. Uguccioni, M. (1995) Monocyte chemotactic protein MCP-2 activates human basophil and eosinophil leukocytes similar to MCP-3. *J.Immunol* 154 (8), 4166-72.

Weeren P, Bruyn K, Vries-Smits A, van Lint J, Bugering B (1998). Essential Role for Protein Kinase B (PKB) in Insulin-induced Glycogen Synthase Kinase 3 Inactivation. *J.Biol.Chem.* 273: 13150-13156

Weng Q-P, Andrabi K, Klippel A, Kozolowski MT, Williams LT Avruch J. (1995) Phosphatidylinositol 3-kinase signals activation of p70 S6 kinase *in situ* through site specific p70 phosphorylation. *PNAS.* 92:5744-5748.

Williams M, Arthur S, Balendran A, van der Kaay J, Poli V, Cohen P, Alessi D. (2000). The role of 3-phosphoinositide-dependent protein kinase 1 in activating AGC kinases defined in embryonic stem cells. *Curr. Biol.* 10:439-448

Wilkinson B, Owen J, Jenkinson E. (1999). Factors Regulating Stem Cell Recruitment to the Fetal Thymus *J Immunol.* 162: 3873-3881.

Witt, D, Lander A.. (1994). Differential binding of chemokines to glycosaminoglycan subpopulations. *Curr. Biol. 4:394.*

Wolscholski R, Kodaki R, McKinnon T, Waterfield M, Parker P. (1998). A comparison of deoxyviridin and wortmannin as inhibitors of phosphoinositide-3 kinase. *FEBS Letters.* 342:109-114.

Wolthius R, Bos J. (1999). Ras caught in another affair: the exchange factors for Ral. *Curr.Opin.Gen.Dev.* 9:112-117.

Wu DQ. Larosa GJ. Simon MI. (1993). G-Protein coupled signal transduction pathways for IL-8. *Science.* 261: 5117. 101-103.

Wymann M,Bulgarelli-Leva G, Zvelebil M, Pirola L, Vanhaesebroek B, Waterfield, Panayotou G. (1996) Wortmannin Inactivates Phosphoinositide 3-kinase by Covalent Modification of Lys-802, a Residue Involved in the Phosphate Transfer Reaction. *Mol.Cell.Biol.* 16:1722-1733.

Wymman M and Pirola L (1998). Structure and function of phosphoinositide 3-kinases. Biochim. *Biochim. Biophys. Acta.* 1436:127-150.

Xiao L, Rudolph S, Owen TJ, Spira TJ, Ial RB. (1998). Adaption to promiscuous usage of CC CXC-chemokine coreceptors *in vivo* correlates with HIV-1 disease progression. *AIDS.* 10:137-143.

Xu Y, Seet L-F, Hanson B, Hong W. (2001). The Phox homology (PX) domain, a new player in phosphoinositide signalling. *Biochem.J.* 360:513-530.

Yamasaki M, Arai H, Ashida N, Ishii K, Kita T. (2001). Monocyte chemoattractant protein 1 causes differential signalling mediated by proline-rich tyrosine kinase 2 in THP-1 cells. *Biochem. J.* 355:751-756.

Yao R, Cooper GM (1995). Requirement for phosphatidylinositol-3 kinase in the prevention of apoptosis by nerve growth factor. *Science.* 267:2003-2006.

Yart A, Laffargue M, Mayeux P, Chretien S, Peres C, Tonks N, Roche S, Payrastre B, Chap H, and Raynal P. (2001). A Critical Role for Phosphoinositide 3-Kinase Upstream of Gab1 and SHP2 in the Activation of Ras and Mitogen-activated Protein Kinases by Epidermal GrowthFactor. *Journal of Biological Chemistry.* 276:8856-8864.

Yatomi Y, Hazeki O, Kume S, Ui M (1992) Suppression by wortmannin of platelet responses to stimuli lead to inhibition of plecktrin phosphorylation. *Biochemical Journal.* 285:745-51.

Youn B, Kim Y, Mantel C, Yum K, Broxmeyer H (2001) Blocking of c-FLIP$_L$-independent cycloheximide-induced apoptosis or Fas-mediated apoptosis by the CC chemokine receptor 9/TECK interaction. *Blood.* 98:925-933.

Yousef S, Maor G, Wilbaum G, Grabie N, Gour-Lavie A, Karin N. (2000) C-C chemokine-encoding DNA vaccines enhance breakdown of tolerance to their gene products and treat ongoing adjuvant arthritis. *J. Clin. Invest.* 106:361

Zhang YJ, Rutledge BJ, Rollins BJ. (1994). Structure/activity analysis of human monocyte chemoattractant protein-1 (MCP-1) by mutagenesis: identification of a mutated protein that inhibits MCP-1-mediated monocyte chemotaxis. *J. Biol. Chem.* 269:15918-15924

Zhang J, Banfic H, Straforini F, Tosi L, Volina S, Rittenhouse S. (1998). A type II phosphoinositide 3-kinase is stimulated via activated integrin in platelets. A source of phosphatidylinositol 3-phosphate. *J. Bio. Chem.* 273:14081-140084.

Zhang W and Neer E. (2001) Reassembly of Phospholipase C-β_2 from Separated Domains - analysis of basal and G protein-stimulated activities *J. Biol. Chem.* 276:2503-2508.

Zhou N, Lou Z, Luo J, Liu D, Hall J, Pomerantz R, Huang Z. (2001) Structural and Functional Characterisation of Human CXCR4 as a Chemokine receptor and HIV Co-receptor by Mutagenesis and Molecular Modelling Studies. *J..Bio. Chem.* (In press)

Zou YR, Kottmann AH, Kuroda M, Taniuchi I, Littman DR: Function of the chemokine receptor CXCR4 in haematopoiesis and in cerebellar development. *Nature* 393:595 - 599, 1998

Zhou Y, Kurihara T, Ryseck RP, Yang Y, Ryan C, Loy J, Warr G, Bravo R: Impaired macrophage function and enhanced T cell-dependent immune response in mice lacking CCR5, the mouse homologue of the major HIV-1 coreceptor. *J Immunol* 160:4018 -4025, 1998

Appendices

Appendix 1 – Tissue Culture

THP-1 cells

RPMI 1640 with 25 mM HEPES

2nM gltamine

100ug/ml penicillin

100 units/ml streptomycin

HEK-293 cells

DMEM

0.1mM MEM non-essential amino acids

2mM gltamine

1mM sodium pyruvate

800μg/ml geneticin (G418) *only for transfectants

Appendix 2 – Western Blotting

Sample buffer

5% SDS

50% glycerol

200mM Tris-HCL (pH 6.8)

5% 2-mercaptoethanol

In sterile filtered H_2O coloured with bromophenol blue

TBS

20mM Tris-base

2mM NaCl

Adjust to pH 7.5

* For TBSN – 0.05% NP40 is added

Blocking buffer (1)

5% BSA

1% ovalbumin

0.05% sodium azide

Blocking buffer (2)

5% Marvel

0.05% sodium azide

Made up in TBS

Running buffer

192 mM glycine

25mM Tris-base

0.1% SDS.

Made up in TBS

Stripping buffer

100mM 2-mercaptoethanol

2% SDS

62.5 mM Tris-HCL

Made up in TBS

Coomassie Blue

0.25% Coomassie Blue (w/v)

45.4% Methanol (v/v)

9.2% glacial acetic acid

Made up to final volume in H_2O

Preparation of acrylamide gels

Component	**7.5%**	**10%**	**Stacking gel**
29.2% Acrylamide/ 0.8% N,N-methylene-bis-acrylamide	3.5	5	1.6
1 *M* Tris 8.8 (ml)	5.6	5.6	-
Water (ml)	5.6	4.35	7.3
1 *M* Tris pH 6.8 (ml)	-	-	3.5
10% SDS (ml)	0.25	0.25	-
10% APS (ml)	0.05	0.05	0.065
TEMED (ml)	0.02	0.02	0.012
Final Volumes	15	15	12.477

Antibodies used for Western blotting.

Assay	Primary antibody		Secondary antibody
	Source	Conc.	Conc.
Phospho-ERK1/2(42/44)	Rabbit	1/1000	1/10,000
Pan ERK-1	Rabbit	1/1000	1/10,000
Pan ERK-1/2	Rabbit	1/1000	1/10,000
Phopsho-JNK(^{Thr}183/^{Tyr}185)	Rabbit	1/1000	1/10,000
Phospho-p38(Thr180/^{Tyr}182)	Rabbit	1/1000	1/10,000
ERK-1	Rabbit	1/1000	1/10,000
pJNK	Rabbit	1/1000	1/10,000
p38	Rabbit	1/1000	1/10,000
Phospho-PKB (^{ser}473)	Rabbit	1/1000	1/2000 (in 5% Marvel/TBS)
Phopsho-GSK-3αβ(^{ser}21/9)	Rabbit	1/1000	1/2000 (in 5% Marvel/TBS)
PKB/Akt	Rabbit	1/1000	1,10,000
GSK-3α	Rabbit	1/1000	1/10,000
PI3K-C2α	Rabbit	1/1000	1/10,000
PI3K-C2β	Rabbit	1/1000	1/10,000
p110γ	Rabbit	1/1000	1/10,000
p85α	Mouse	1/1000	1/20,000
Antiphophotyrosine (4G10)	Mouse	1/10,000	1/20,000
Gab2	Rabbit	1/1000	1/20,000
Ras (p21)	Mouse	1/1000	1/4000
Anti-myc (9E10)	Mouse	1/10,000	1/4000

Appendix 3 – Lipid kinase assay

Lipid kinase buffer

5mM $MgCl_2$

0.25 mM EDTA

20mM HEPES

pH – 7.4

TLC solvent
Propan-1-ol, glacial acetic acid, water [130:4:66(w/v)]

Appendix 4 – RT-PCR

Primers for chemokine receptors

Human gene	**Product length (bp)**	***Sequence***	
GAPDH	178	Sense: Antisense:	5' GACATCAAGAAGGTGGTGAA3' 5'TGTCATACCAGGAAATGAGC3'
β-Actin	176	Sense: Antisense:	5'GACCCCTTCATTGACCTCAAC3' 5'GCAGTAATCTCCTTCTGCATC3'
CCR1	327	Sense: Antisense:	5'ACCTGCAGCCTTCACTTTCCTCAC3' 5'GGCGATCACCTCCGTCACTTG3'
CCR2	255	Sense: Antisense:	5'CCAACTCCTGCCTCCGCTCTA3' 5'CCGCCAAAATAACCGATGTGATAC3"
CCR3	315	Sense: Antisense:	5'TGGCGGTGTTTTTCATTTTC3' 5'CCGGCTCTGCTGTGGAT3'
CCR4	349	Sense: Antisense:	5' GAAGAAGAACAAGGCGGTGAAGAT3' 5' TTCTGAACTTCTCCCCGACAAA3'

Appendix 5. Biochemical reagents

β-glycerophosphate	Sigma, UK
[^{32}P]-ATP	Dupont NEN, USA
[^{32}P] –orthophosphoric acid	Dupont NEN, USA
[^{3}H] PtdIns (4)P	Amersham International, UK
[^{3}H] PtdIns (4,5)P_2	Amersham International, UK
[^{3}H] – palmitic acid	Amersham International, UK
2-mercaptoethanol	Sigma, UK
Adenosine triphoshate	Sigma, UK
Ammonium persulphate	BDH, UK
Ammonium Phosphate	Sigma, UK
Anti-Gab2	Cell Signalling Technologies, USA
Anti-PKB/Akt	Cell Signalling Technologies, USA
Anti-P38 antibody	Upstate Biotech. USA
Anti-CCR2 – PE conjugate	R&D systems, UK
Antiphosphotyrosine (4G10)	Upstate Biotech. USA.
Biorad 'Bradford' reagent	Biorad, UK
Bovine serum albumin	Sigma, UK
Bromophenol blue	BDH, UK
Calcium chloride	Sigma, UK
Chloroform	BDH, UK
Coomassie blue	Sigma, UK
Digitonin	BDH, UK
DTT	Promega, UK
EDTA	Sigma, UK
EGTA	Sigma, UK

Enhanced Chemiluminescence Reagent	Amersham Internation, UK
Ethyl Formate	BDH, UK
Flowscint IV Scintillation Cocktail	Canberra Packard, UK
Foetal bovine serum	Gibco BRL, Scotland
Folsh Lipids	Sigma, UK
Fura-2/AM	Calibiochem, UK
Geneticin (G418)	Gibco BRL, Scotland
GF/A filter paper	Whatman, UK
Glacial acetic acid	BDH, UK
Glycerol	Sigma, UK
Goat anti-mouse peroxidase conjugate	Santa Cruz, UK
Goat anti-rabbit peroxidase conjugate	Santa Cruz, UK
Hank balanced salt solution	Gibco BRL, Scotland, UK
HEK 293 human D6 transfectants	Gift from R. Nibbs, Glasgow, UK
HEPES (1 M liquid)	Gibco BRL, UK
HEPES (solid)	Gibco BRL, UK
Hydrochoric acid	BDH, UK
Iodine	Sigma, UK
Iodoacetamide	Sigma, UK
Leupeptin	Sigma, UK
Linear K Preadsorbent TLC plate	Whatman, UK
Lithium chloride	Sigma, UK
LT-82 (lethal toxin)	Gift from M Popoff, Institut Pasteur, Paris
LT-9048 (lethal toxin)	Gift from M Popoff, Institut Pasteur, Paris
Magnesium chloride	Sigma, UK
MCP-1	R&D Systems, UK
MCP-2	R&D Systems, UK
MCP-3	R&D Systems, UK
MCP-4	R&D Systems, UK
Methanol	BDH, UK
MIP-1α	Peprotech, Rocky Hill, USA
M – MLV Reverse transcriptase	Promega, UK
Molecular weight markers	Gibco BRL, UK
MRNA QuickPrep Isolation kit	Pharmacia, UK
N-butanol	BDH, UK
NP-40	Fisons, UK
Optiphase Scintillation fluid	Canberra Packard, UK
Orthophosphoric acid	BDH, UK
p85α antibody	Wyman, UK
p110γ antibody	Santa Cruz, USA
Pan ras antibody	Santa Cruz, USA
Penicillin/Streptomycin	Gibco BRL, UK
Pepstatin	Sigma, UK
Pertussis Toxin	Sigma, UK
Petroleum Ether	BDH, UK
Phosphate buffered saline	Sigma, UK
Phosphatidylinositol	Sigma, UK
Phospho-ERK1/2 antibody	New England Biolabs, USA
Phospho-GSk-3$\alpha\beta$ antibody	Cell Signalling Technologies, USA
Phospho-JNK/SAPK antibody	Cell Signalling Technologies, USA
Phospho-P38 antibody	Cell Signalling Technologies, USA
Plasticware (tissue culture grade)	Nunc, UK

PMA	Sigma, UK
PMSF	Sigma, UK
Potassium oxalate	Sigma, UK
Propan-1-ol	BDH, UK
Protein G beads	Sigma, UK
Roche compound (Ro-320432)	Calibiochem, UK
RNAsin Ribonuclease Inhibitor	Promega, UK
RNAzol™B	Biotex, USA
RPMI 1640	Gibco BRL, UK
Sodium azide	Sigma, UK
Sodium chloride	Sigma, UK
Sodium dodecyl sulphate	Sigma, UK
Sodium hydroxide	Fisons, UK
Sodium othovanadate	Sigma, UK
TEMED	Sigma, UK
Tetrabutylammoniumhydrogen sulphate (TBAS)	Sigma, UK
THP-1 cells	ECCAC, UK
Tris-Base	Sigma, UK
Tris-HCL	Sigma, UK
Tween 20	Sigma, UK
Versene	Gibco BRL, UK
Wortmannin.	Sigma, UK
X-OMAT film	Amersham International, UK.